CURRENT TRENDS OF INSECT PHYSIOLOGY AND POPULATION DYNAMICS: MODELING INSECT PHENOLOGY, DEMOGRAPHY, AND CIRCADIAN RHYTHMS IN VARIABLE ENVIRONMENTS

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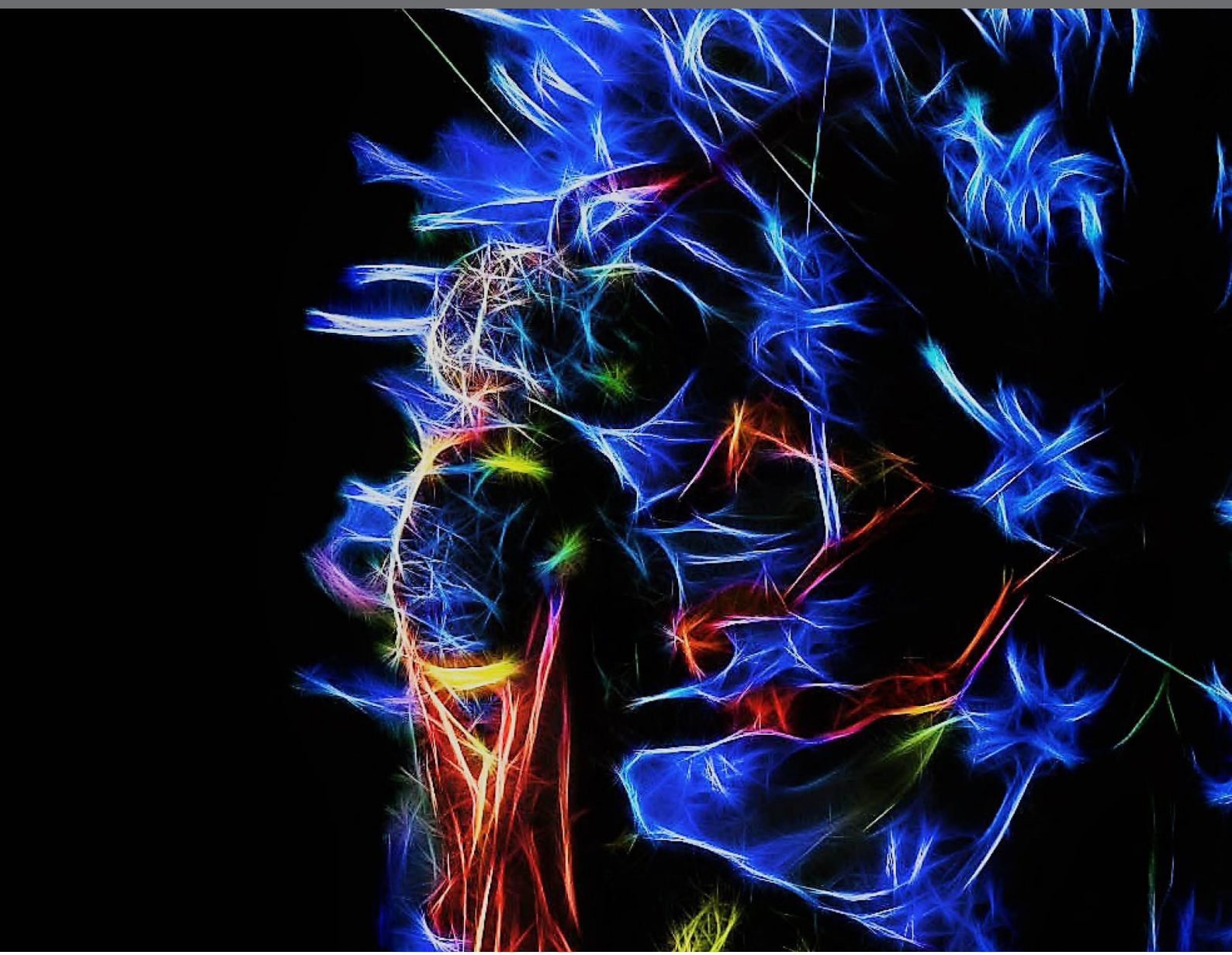

frontiers Research Topics

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CURRENT TRENDS OF INSECT PHYSIOLOGY AND POPULATION DYNAMICS: MODELING INSECT PHENOLOGY, DEMOGRAPHY, AND CIRCADIAN RHYTHMS IN VARIABLE ENVIRONMENTS

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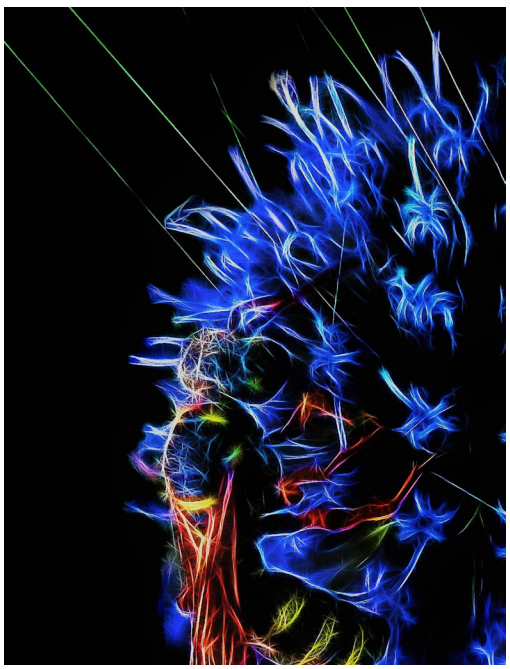

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The current eBook collection includes substantial scientific work in describing how insect species are responding to abiotic factors and recent climatic trends on the basis of insect physiology and population dynamics. The contributions can be broadly split into four chapters: the first chapter focuses on the function of environmental and mostly temperature driven models, to identify the seasonal emergence and population dynamics of insects, including some important pests. The second chapter provides additional examples on how such models can be used to simulate the effect of climate change on insect phenology and population dynamics. The third chapter focuses on describing the effects of nutrition, gene expression and phototaxis in relation to insect demography, growth and development, whilst the fourth chapter provides a short description on the functioning of cir cadian systems as well as on the evolutionary dynamics of circadian clocks.

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EDITORIAL
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Editorial: Current Trends of Insect Physiology and Population Dynamics: Modeling Insect Phenology, Demography, and Circadian Rhythms in Variable Environments

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Editorial on the Research Topic

Current Trends of Insect Physiology and Population Dynamics: Modeling Insect Phenology, Demography, and Circadian Rhythms in Variable Environments

Physiological processes of insects and related arthropods are strongly affected by abiotic factors in which temperature and photoperiod have a predominant function setting the limits of distribution in time and space. Other important factors, which may affect the development of insect populations, for a particular environment, may be related to alterations in humidity levels and type of available nutrition. In temperate climates, seasonal changes of the above environmental conditions may cause alterations in physiological processes which are further expressed at the macroscopic level through alterations in population dynamics and insect phenology.

Traditionally, such processes are described using certain type of models including empirical and conceptual modeling descriptions, which in their simplest forms are based on fundamental rules of temperature-dependent development. Recently, as we include more variables and proceed to more complex physiological systems make such models less accurate. As a result, several theoretical and experimental works have been carried to shade light on how physiology and population dynamics is affected by a particular environment. Additionally, when merged with current progresses in modeling and computation they offer new means in describing insect physiology and population dynamics.

In the present Research Topic in Frontiers in Invertebrate Physiology we embody a series of original works which highlight some of the current directions of this field. There is a great diversity of modeling studies included that goes across several insect taxa and ecosystems reflecting the magnitude of the effects of the physical environment on insect physiology and related population processes. However, most studies included take into account the impact of principal abiotic factors (temperature and photoperiod) as driving forces and use models (empirical, computational,

conceptual and other) as a basic tool to describe and predict their effects on the physiological functioning at the macroscopic-population, or, cellular-individual levels.

One of the major challenges of modeling insect population dynamics is not only to include the effects of climates but also the importance of landscape complexity. Lux et al. are using the medfly (*Ceratitis capitata*) as a model organism, propose a "virtual farm" modeling concept for enhancement of site-specific pest management and validates its utility for Integrated Pest Management (IPM). In the same context, Lux in a method article, extends the modeling approach to gain insight into the environmental and landscape factors driving insect behavior and invasion dynamics and demonstrates *in silico* that incipient cryptic populations may occasionally occur in a territory despite that local climate and a fragmented landscape may be sub-optimal for their establishment.

Joshi et al. are using degree-day (DD) phenology models to predict the timing of the codling moth (*Cydia pomonella*) development and phenology events and illustrate the continuous need for validating the functionality of available degree-day models when applied under new environmental conditions. They also bring in to account the potential impact of changes in orchard environment and crop management practices on the biology and phenology of the species.

Another constraint in traditional degree-day models is that they ignore the life-history functions that are not primarily influenced by temperature, such as the transition between diapausing and non-diapausing life stages. In this direction, Nielsen et al. tackles the constrain of the utility of single parameters DD models and have developed a stochastic agent-based model for insects that overwinters as diapause adults and further describe the stage specific phenology and population dynamics of the insect species *Halyomorpha halys* across a variety of geographic regions. It is shown that agent-based models better approximate the phenology of a species that has strongly overlapping life stages and heterogeneity in its population traits.

Moreover, although a large body of literature is devoted to the development of temperature driven phenology models, yet the relevance of fine-scale climatic data and their effect on accurate modeling species population dynamics and its distribution remains a matter of debate. The study of Rebaudo et al. supports that in the absence of microclimate dataset, global climate models are best fitted to predict species abundances at large scales (and low settlement), while microclimate datasets best predict abundances at fine scales (and high resolution). Furthermore, Robinet et al. discuss how climate change could alter phenology in the pine processionary Moth, an emblematic species, and how climate spatial heterogeneity interacts with phenology, making the mechanism of range expansion more complex than initially thought.

Insect life cycle demography and related physiological process of constant climate may be likewise affected by the quality and quantity of nutrition, which is available as well as on other factors such as the presence of a light source. Thus, two papers deal with the effects other factors than temperature on the life cycle and certain demographic aspects of insects. In the first related study, Kouloussis et al. have developed a feeding quantification method for the medfly and have brought us a better understanding of the nutritional influences on medfly lifespan and its interplay with reproduction and provide certain parametric and non-parametric approaches. To determine the underlying mechanisms of insect phototactic behaviors Zhang et al. recorded the phototactic response of two morphs of the pea aphid *Acyrthosiphon pisum* in different nymphal instars to white. They have further analyzed the relationship between phototactic response and fecundity and conclude that that light signals are important for aphid dispersal and distribution, and are also essential for the pea aphids to cope with environmental changes.

The two following contributions deal with the outcome of specific external causes on the degree of different types of gene expression aspects. The first work of Kola et al. aimed to examine the effect of dsRNA molecules on silencing the genes cytochrome *CYP6* and aminopeptidase *APN* of the rice yellow stem borer (YSB), *Scirpophaga incertulas* by feeding larvae on dsRNA treated cut stems. It is indicated that larvae fed with dsRNA specific to the CYP6 and APN showed significant inhibition of maturation in a time dependent manner, a trend which may bear some interesting facets of pest management in affecting their populations. On the other hand, to develop more realistic predictions about the biological impact of climate change in biological invasions, Boher et al. have assessed the combined effects of the mean and the variance of temperature on the physiological tolerance in adults of the invasive fruit fly *Drosophila melanogaster* and the native *Drosophila gaucha*. Through the expression of heat shock protein (*hsp90*) as a biochemical response they suggest that historical biogeography may be an associated important feature of species under current and future variable climatic scenarios.

The two final review papers included focus on the description of insect circadian rhythms with emphasis to insect circadian clocks. The review of Ito and Tomioka provides a description of the heterogeneous nature of peripheral circadian clocks in the fruit fly *D. melanogaster* and their dependence on the central clock, and discuss their significance in the temporal organization of physiology in peripheral tissues/organs. They bring out new lines of evidence that clearly demonstrate that the oscillatory machinery and degree of independence from the central clock vary among the peripheral clocks and emphasize that we should pay attention to environmental cues other than light. Finally, Rivas et al. put emphasis on the molecular mechanisms and evolutionary dynamics of insect clocks in variable environments. Circadian rhythms may be expressed as oscillations in behavior, metabolism and physiology, that have a period close to 24 h and are controlled by an internal pacemaker that evolved under strong selective pressures imposed by environmental cyclical changes, mainly of light and temperature. They include additionally a conceptual model of the circadian clock in *Drosophila*, which depicts multiple interlocked loops that are affected by temperature and light/dark duration suggesting that light and temperature entrainment have a basic role.

Overall, the current Research Topic includes method articles, original research articles and reviews, which contribute toward a better understanding of modeling certain features of insect physiology at the population or individual level. Additionally, we feel that the current collection may be a motive for future research directions in studding insect physiology, demography and circadian rhythms in variable environments.

AUTHOR CONTRIBUTIONS

PD proposed and organized the research topic. PD, SS, and AR, have made substantial, direct and intellectual contribution to the editorial, and approved it for publication.

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METHODS
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Validation of Individual-Based Markov-Like Stochastic Process Model of Insect Behavior and a "Virtual Farm" Concept for Enhancement of Site-Specific IPM

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The paper reports application of a Markov-like stochastic process agent-based model and a "virtual farm" concept for enhancement of site-specific Integrated Pest Management. Conceptually, the model represents a "bottom-up ethological" approach and emulates behavior of the "primary IPM actors"—large cohorts of individual insects—within seasonally changing mosaics of spatiotemporally complex faming landscape, under the challenge of the local IPM actions. Algorithms of the proprietary PESTonFARM model were adjusted to reflect behavior and ecology of *R. cerasi*. Model parametrization was based on compiled published information about *R. cerasi* and the results of auxiliary on-farm experiments. The experiments were conducted on sweet cherry farms located in Austria, Germany, and Belgium. For each farm, a customized model-module was prepared, reflecting its spatiotemporal features. Historical data about pest monitoring, IPM treatments and fruit infestation were used to specify the model assumptions and calibrate it further. Finally, for each of the farms, virtual IPM experiments were simulated and the model-generated results were compared with the results of the real experiments conducted on the same farms. Implications of the findings for broader applicability of the model and the "virtual farm" approach—were discussed.

Keywords: *Rhagoletis cerasi*, European cherry fruit fly, virtual farm, agent-based models, site-specific IPM

INTRODUCTION

European regulations stipulating integrated pest management (IPM) (Directive 2009/128/EC[1]), scarcity of robust and economically sound IPM methods and dwindling lists of the approved pesticides (European Commission, DG SANCO, 2013)—combined—render the fresh production increasingly challenging. The problem is particularly acute in spatiotemporally complex medium scale farming systems typical for "ecologically-conscious" non-industrial

[1] Directive 2009/128/EC of the European Parliament and of the Council of 21 October 2009. Official Journal of the European Union L309/71, 24.11.2009 http://www.eppo.int/PPPRODUCTS/information/2009_0128_EU-e.pdf

fruit and vegetable production, where generic IPM protocols are seldom effective without site-specific adaptation. Indeed, the local IPM performance is determined by the local farm topography and traits, which shape the outcome of fine interplay among concurrent processes, where the on-farm dwelling cohorts of individual, independently operating insects are the key causative actors. The latter merits a "bottom-up" and individual-focused "ethological" approach (Lux, 1992, 1994; Lux and Gaggl, 1996), and application of Markov-like processes in individual-based stochastic models (Lux, 1989, 2014; Grimm et al., 2005). Through inclusion of the site-specific spatiotemporal features, such models could serve as a "virtual farm" emulating the key processes determining performance of the local farming system, and offer quantified insights into the mechanisms driving the local IPM performance.

Such individual- and/or agent-based models, enacting complex processes by the actions of their key "virtual actors," offer unprecedented flexibility and heuristic advantages (Fajardo, 2009). For this reason, the agent-based modeling and the concept of "virtual environmental laboratories" is increasingly applied in studies on complex systems in ecological and evolutionary research (DeAngelis and Mooij, 2005; Jovani and Grimm, 2008; DeAngelis and Grimm, 2014), development of environmental management (Reed et al., 2016) and decision making tools (Grimm et al., 2014), land management and urban planning (Parker et al., 2002; Parker, 2005). Regrettably, in the domain of horticulture and the IPM, the potential of such approaches still remains largely unrecognized and underutilized.

Our objective was to explore the potential of an agent-based "virtual farm" approach for simulation of the local IPM experiments, and "virtual" assessment of the net effects of multiple, concurrent modifications introduced into the local system. The paper reports approaching such task with application of the PESTonFARM model (Lux, 2014), adapted to the cherry growing system and its key pest—the European cherry fruit fly, *Rhagoletis cerasi*. To accomplish this task, our "interim" aim was to assemble and categorize scattered, published and "gray," knowledge about ecology and behavior of the target pest (*R. cerasi*), and supplement it with auxiliary on-farm observations and experiments. Afterwards, encapsulate the pertinent information into model procedures and parameters, and convert it into an operable tool suitable for the local IPM enhancement. In the model, actions and fate of the virtual individuals enacting the IPM process (*R. cerasi* females) are stochastically determined by the assigned set of behavioral rules and parameters, and modulated by the status of the location (farm sector) of their actual residence. Farm topography and traits are represented by set of grids, with quantified sectors, which values fluctuate during the season, according to plant phenology, IPM treatments etc.

Practical application of the model, as a site-specific IPM optimization tool—was intended from the onset. Alike, converse use of the model for adjustment of the local agro-landscape and designing "pest resilient" farm topographies. Our focus was to obtain assessments for the units relevant to the end-user, such as plots containing various cherry cultivars, IPM treatments, sets of monitoring traps, etc. Although, the model simulates all the processes for each farm (grid) sector, *R. cerasi* typically occurs at very low densities, thus the numbers generated daily for each sector tend to be erratic and of limited practical interest. Our pragmatic goal was to use the model rather for development of typical site-specific IPM tactics, optimized for the local farm topography and the locally prevailing climatic conditions, rather than adjusting the on-farm decisions to the weather fluctuations of a particular season (although such application is also conceivable).

Concise presentation of the agent-based models presents a challenge. They can be characterized by their purpose, description of causative agents, the set of rules and interrelations taken into account, assumptions and estimates of the key parameters, etc. (Grimm and Railsback, 2005). But unlike mathematical models composed of formal, explicit, and easy to scrutinize equations with closed form solutions describing changes in the studied system, the rule-based simulation models are strictly focused on active emulation of interactions among the individual causative actors (agents) and the system (An et al., 2009). The "end result" of the simulation process is neither determined nor programmed, each time it "emerges" *de novo*—generated by the activity of its agents'. Inadequacy of established terminology and universally accepted practice for presentation and testing ecological models (Grimm and Railsback, 2005)—compounds the difficulty even further. Nonetheless, growing popularity of the agent-based modeling fostered advancements in implementation of the presentation standards (Grimm et al., 2006, 2010, 2014; Polhill et al., 2008), and thus the model description largely follows the ODD (overview, design, concepts and details) protocol proposed and updated by Grimm et al. (2006, 2010).

Further to the outline of the model, its parameterization and on-farm validation, a few examples of its potential for IPM enhancement are discussed.

METHODS

Outline of the Model

PESTonFARM (Lux, 2014) is a proprietary, site-specific agent-based model, implemented in the Visual Basic for Applications (VBA) (MS Office for Mac 2011) and fully integrated with the commonly used MS Excel. In the reported study, recently enhanced generic 3.1 version of the PESTonFARM model was used, which can emulate behavior and development of multivoltine pests with multiple, overlapping generations and age-cohorts, operating within seasonally fluctuating mosaics of the local farming landscape, according to the local weather conditions. It reflects farm topography and its key features, emulates host-plant phenology, and behavior of the local pest population during a "virtual" IPM experiment. Upon each run, it generates a unique, but stochastically equivalent set of projections/results presented in the formats resembling real on-farm experiments. All on-farm phenomena are simulated with 1-day temporal resolution. Spatial resolution is determined by pest biology and its estimated daily mobility ranges. The model consists of two main modules: "virtual insect" and "virtual farm."

Virtual insect module: The "virtual insect" module encapsulates relevant information about insect ecology and behavior, and accordingly, determines (in a stochastic sense) behavior of individual "virtual" insects—members of cohorts representing the local pest population. Insect behavior is assumed to resemble a Markov-like process, each behavioral step, event or "decision" of each individual "virtual" insect is fully randomized and stochastically dependent on its age, weather conditions, current status of the sector of its actual residence, and where relevant—also that of the nearby sectors.
Virtual farm module: The "virtual farm" module encapsulates relevant information about the particular farm/site to be modeled, and constitutes "virtual environment" which determines development and behavior of the local "virtual insect" population. Various farm aspects are represented by dynamic matrices of quantified square sectors, which values fluctuate daily throughout the season according to the local host phenology, spatiotemporal efficacy profiles of the applied IPM treatments, and the changes imposed by actions of the "virtual insects" themselves.

The key parameters and relations adopted in the model are outlined in **Table 1**, while their biological background is discussed under the results section. More detail model description, according to the ODD (overview, design, concepts and details) protocol proposed and updated by Grimm et al. (2006, 2010), is provided in the Complementary materials.

Identification of the Relevant Aspects to *R. cerasi* Biology, and Model Adaptation

The generic model, PESTonFARM v. 3.1, was adapted to reflect the key aspects of *R. cerasi* biology. A catalog of the relevant behaviors and on-farm processes was identified based on review of the published information and gray literature, extrapolations from closely related species, compiling experiences of the authors', and the results of our *ad-hoc* on-farm observations and auxiliary experiments. Raw results of the "historic" experiments conducted on-farm in the past (JKI, BOKU, PC-Fruit) were used for calibration of the model algorithms, in particular parameters of its "virtual insect" module. Afterwards, the model was "locked" and subject to on-farm validation without any further adjustments to its internal parameters.

The main biology aspects identified and used for the model adaptation, assumptions and the basis for estimation of the key parameters—are presented in the "Results" section, while the specific model assumptions, derived processes and adopted parameters are presented in **Table 1**.

Model Validation

Model validation was conducted on three sweet cherry farms located in Germany, Austria and Belgium. For each farm, a customized "virtual farm" module was prepared, reflecting its key spatiotemporal features, which was used to simulate the IPM experiments "really" conducted on the farm, and also additional "hypothetic" IPM scenarios. For each IPM scenario modeled, simulation was replicated five times, and its average results were compared with the experimental data collected on the real farms. All five replicates were made with the same initialization settings, describing the experimental assumptions, status and spatiotemporal properties of the respective farms and IPM treatments. Before any comparisons with the experimental results, homogeneity of the simulated replicates was tested and confirmed.

Capacity of the model to reproduce the mark-recapture experiment (dynamics of the re-capture process and patterns of relocations among farm structures) for each pest age category, and the field results (spatial and temporal patterns of trap catches during pest monitoring, fruit infestation patterns, effects of the local pesticide application etc.)—was treated as an evidence of the correct model calibration and validity.

On-Farm Experiments

Experimental Locations

The presented research were conducted on the following locations: (1) Julius Kuhn Institute (JKI) farm, Dossenhem, Germany (N 49°26′ 56.1063″; E 8°38′ 22.3636″; 115 m altitude), (2) University of Natural Resources and Life Sciences (BOKU) farm, Vienna, Austria, (N 48°17′19″; E 16°25′43″; 162 m altitude), (3) Proefcentrum Fruitteelt VZW (PC-Fruit), Metsterenweg, Belgium, (N 50°50′34″; E 5°10′24″; (4) WULS-SGGW campus, Warsaw, Poland, (N 52°10′03″; E 21°02′44″; 101 m altitude). The locations are referred to as follows: JKI farm, BOKU farm, PC-Fruit farm, and WULS campus, respectively. The overall topography of the farms selected for modeling, arrangement of the plots containing sweet cherry trees and positions of the monitoring traps (Rebel) are presented on **Figure 1**.

Insect Material

Insect collections and treatment followed methodology of Köppler et al. (2010). Larvae of *R. cerasi* were obtained from field-infested cultivated sweet cherries collected in the experimental orchards of JKI farm and from wild cherry trees growing at WULS campus. The collected larvae pupated within a few hours, and the pupae were kept at room temperature for ca. 8 weeks. Afterwards, to facilitate obligatory diapause, the pupae were stored for at least 195 days at 4°C, 65% ± 5 RH, and kept as a "cold stock" in such conditions for up to 11 months.

To initiate post-diapause development and eclosion, the required numbers of pupae were randomly selected from the "cold stock" and transferred for 25–30 days to climatic chambers set at 25/18°C day/night, respectively, 65 ± 5% RH and L16/D8 photoperiod. After eclosion, both male and female flies were transferred to larger BugDorm cages (30 × 30 × 30 cm) and kept in the same ambient conditions. Males and females were either mixed or kept separately, according to experimental needs. Each cage was supplied with water and food sources (dry yeasts mixed with sucrose, 1:4), offered on Petri dishes *ad libitum*. Water was offered on a wet piece of sponge, with one end submerged in a 100 ml container filled with water, and the other end protruding through a hole in the container's lid. Water was changed daily, while food every third day. Whenever, flies of various age categories

TABLE 1 | The main aspects of biology, key processes taken into account, and adopted parameters.

Aspect	Process/Parameter	Adopted values	Relation/sub-model	Basis/Source
Adult females	Sex ratio of adults emerging in spring	1:1	Constant	Assumed, based on Łęski, 1963; Daniel and Grunder, 2012
	Pattern of adult emergence in spring	Staggered, lasting 35–50 days, with 70–90% emerging during peak 14 days	Bell-shaped function adjusted to fit the published data, adjusted to the local farm conditions	Vogt et al., 2010, historic data, aver. temperatures prevailing in spring in each location
	Lifespan (average under optimal conditions and in absence of extrinsic mortality causes)	59 days	Constant	Assumed, based on: Köppler et al., 2008; Moraiti et al., 2012
	Maximum modeled individual lifespan	95 days	Constant	
	Intrinsic age-dependent adult daily mortality risk	Daily average calculated according to cohort age	Gompertz function adjusted to fit published data	
	Extrinsic daily mortality risk caused by complex of on-farm resident predators and natural enemies	3%	Constant (except the areas of pesticide application, with transient suppression of the local natural enemies)	Broadly estimated, based on analysis of historic data
Immature stages	Status of eggs	Fertilized (100%)	Constant	Assumed
	Sex ratio	1:1	Constant	Assumed
	Duration of in-fruit development (from egg to mature larva jumping out from the fruit for pupation)	20–23 days	Constant, adjusted to the locally prevailing temperatures	Assumed based on: Daniel and Grunder, 2012; Łęski, 1963; Vogt et al., 2010
	Combined mortality from egg till the adult emerging next spring	92%	Constant	
Fecundity	Mating status of mature females	Mated (100%)	Constant	Assumed
	Potential lifetime fecundity (under optimum conditions, unlimited availability of food and suitable fruit, absence of extrinsic mortality causes)	365 eggs/female	Constant	Assumed based on: Köppler et al., 2008; Moraiti et al., 2012
	Batch size	1 egg/fruit	Constant	Assumed based on: Daniel and Grunder, 2012 Based on: Köppler et al., 2008; Moraiti et al., 2012
	Intrinsic age-dependent daily fecundity	Range: 0–10, daily average calculated according to cohort age, individual values generated assuming normal distribution	Asymmetric bell-shaped function adjusted to fit published data	
Mobility	Area covered during a single local exploration errand	100 sqm	Constant	Assumed based on: preliminary on-farm observations, and Böckmann et al., 2012, 2014; Daniel and Grunder, 2012; Daniel and Baker, 2013
	Farm sector size	100 sqm (10 × 10 m)	Constant	Adopted to fit insect's local exploration range
	IN/OUT balance between emigration from the farm and immigration from the neighborhood	1:1	Constant	Assumed for all scenarios presented in the paper
	Micro-migration	Range 30–300 m, potential daily average and SD calculated according to cohort age, individual values generated based on average and SD	Custom-build age-dependent functions	Based on mark-recapture experiments, and Boller, 1969; Łęski, 1963; Wiesmann, 1935; Vogt et al., 2010

(Continued)

TABLE 1 | Continued

Aspect	Process/Parameter	Adopted values	Assumed relation/sub-model	Basis/Source
Cherry phenology, fruit suitability and infestation	Cherry phenology	Flowering time, beginning of fruit suitability, harvest	Cultivar-specific	Recorded on farm
	Fruit suitability for oviposition	From the point of hue change (green to yellowish-green) till harvest	Typically: 31–44% of the average flowering-to-harvest period	Vogt et al., 2010, on-farm records of cultivar phenology
	Daily fruit attractiveness and suitability for larval development	Ranging from 0 to 100%, maximum same for all cherry cultivars	Asymmetric bell-shaped function, max. 1/3 of the fruit suitability period	Assumed, function adjusted to fit on-farm recorded cultivar phenology
	Post-infestation fruit recovery time (if egg or young larva was killed e.g., by a systemic pesticide)	5 days, counted from the day of the egg deposition	Constant	Estimated, based on preliminary observations
	Pre-harvest "concealed" fruit injury, when *de facto* infested fruit still appears unblemished to the consumer.	4 days, counted from the day of the egg deposition	Constant	Recorded on farm
Niche utilization	Fruit infestation [%]	Actual for each sector	Custom build functions, according to type of behavior, with minor impact at low to moderate infestation level	Estimated, based on preliminary observations and analysis of previous (historic) trapping data
	Local population density	Actual for each sector		
Aspect	**Process/Parameter**	**Adopted values**	**Relation/sub-model**	**Basis/Source**
Monitoring with Rebel traps	The effective trapping area surrounding Rebel trap	100 sqm (10 × 10 m)	Constant	Estimated, based on preliminary observations and analysis of previous (historic) trapping data
	Responsiveness of females to Rebel trap	Age dependent, ranging from the initial 80%, to 100% at peak, and declining to 40% afterwards	Asymmetric bell-shaped function adjusted to fit the assumed thresholds	
	Average daily trapping risk for a new Rebel trap, within the range of its activity	5%	Constant	
	Daily decline in trap original trap efficacy, due to dust etc.	1%	Constant	
Pesticide application	Only the insects present in or entering pesticide zone were deemed exposed to additional mortality risks	Daily mortality risk dependent of estimated residual pesticide effectiveness	Custom-build functions	Estimated, based on: Lazić et al., 2014, and producer's application guidelines
	In the areas of pesticide application, transient suppression of the locally resident natural enemies occurs	Daily recovery rate of the natural enemies dependent of estimated residual pesticide effectiveness	Custom-build functions	
Weather impact	Temperature threshold for mating	15°C	Constant	Daniel and Grunder, 2012; Katsoyannos, 1979
	Temperature threshold for oviposition	16°C	Constant	Boller, 1966; Daniel and Grunder, 2012
	Conditions for explorative activity	No rain, temperature > 13°C, wind < 12 m/s, sunshine > 100 W/m^2	Custom-build functions	Estimated, based on observations, and Boller, 1966; Daniel and Grunder, 2012; Katsoyannos, 1979

were required concurrently, batches of pupae were treated as above in a staggered manner, accordingly. Age classes were assembled by random selection of active and "healthy looking" flies with undamaged wings from the respective batches.

On-Farm Dispersal

The Mark-Recapture experiment was conducted at JKI, using insects from JKI stock. Cohorts of 5-, 14-, and 28-day-old females (ca. 300 females each) were concurrently released from the same point. Each individual was marked with a spot (ca. 1 mm diam.)

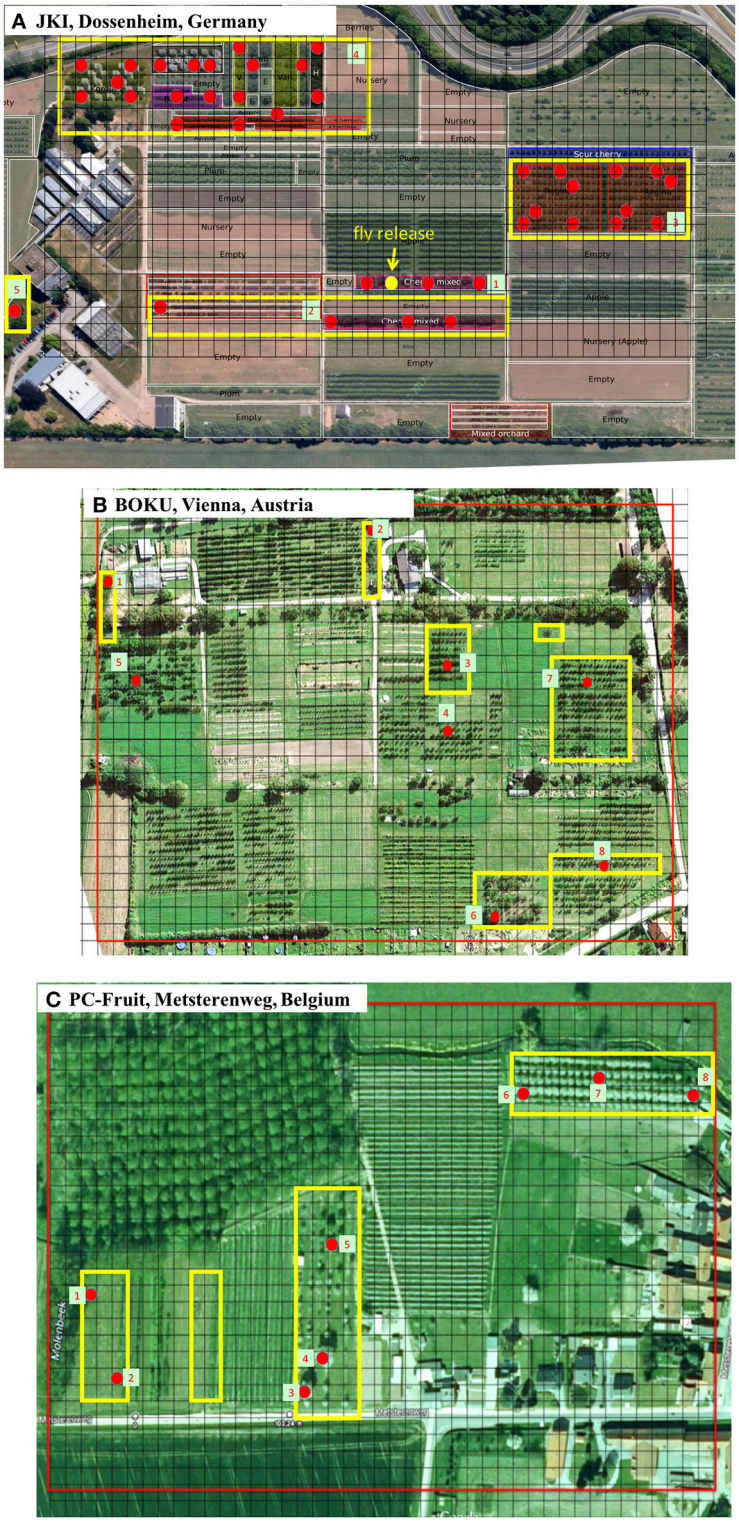

FIGURE 1 | Outline of the experimental farm topography. Red frames: area selected for modeling, Yellow frames: plots containing sweet cherry trees, Red dots: position of the Rebel traps, Grid: farm sectors equivalent to 10 × 10 m on the ground, Background map data: Google Maps.

painted on the thorax, different color for each age category. To intercept the flies relocating within the farm, an array of 38 Rebel traps was established. The traps were checked daily, and numbers of the marked flies trapped were recorded. The experiment lasted 15 days until no more marked flies were caught. Farm topography is presented on **Figure 1A**. Each of the five re-capture zones contained plots of fruiting sweet cherries and a set of Rebel traps. The release zone (1) consisted of a single elongated plot of fruiting cherry trees (mixed cultivars) with three Rebel traps. Adjacent zone (2) comprised parallel plots with mixed cultivars and four traps, separated from the release zone by only a band (ca. 20 m wide) of empty field. Zone three, consisted of a large plot of Regina cultivar with 12 traps, with its center 147 m and its closest edge 90 m apart from the release point, separated from the release zone by a plot of apple trees. Zone four was the largest, contained several cherry plots with various cultivars and 19 traps, its center was 191 apart from the release zone. The last, fifth zone comprised only two large "wild" cherry trees, growing 238 m from the release point. The remainder of the farm contained a number of plots with apple or plum trees, spaced by several empty fields.

Potential Impact of Parasitoids on *R. cerasi* Population

Two samples of pupae, originating from the JKI and WULS stocks, were used. Both samples originated from plots with a history of no recent pesticide application. After 165 days of diapause and cold storage, followed by 21 days of post-diapause development in climatic chambers, numbers of parasitoids emerging from the pupae were recorded daily. The parasitoids were identified by Prof. Kees van Achterberg, Naturalis Biodiversity Center, Leiden, The Netherlands.

IPM Experiments

The IPM experiments were conducted on two farms (BOKU, & PC-Fruit farms), each containing several plots of various sweet cherry cultivars, various non-host fruit trees and plots with no trees (**Figures 1B,C**). The farms varied substantially in terms of latitudinal position and climatic conditions, size, spatial arrangement, tree structure, cultivar composition, and management practice. On each farm, usual IPM operations were conducted, supplemented by pest monitoring. On each farm, the locally established populations of *R. cerasi* were present, greatly varying in population density among the farms. Each on-farm experiment comprised the following steps:

a. *Spatial farm characterization*: A satellite picture of each farm was used for preliminary farm characterization. On each farm, a grid of square sectors (equivalent to 10 × 10 m on the ground) was superimposed and a rectangular "modeling" area (marked by red frame on the farm map) was selected, ca. 9 and 12 hectares, BOKU and PC-Fruit, respectively. Every grid sector was individually characterized by: presence or absence of tree canopies, their average diameter, degree (%) of land coverage by the canopy, dominant species of non-host trees or cultivar of host trees, row direction etc. Afterwards, detailed surveys were conducted on each farm to verify the information "on-the-ground". Based on the collected information, for each farm, a customized farm-representing module was prepared, describing the key spatiotemporal farm features such as crop phenology and distribution patterns, tree canopy size, coverage and row directions, non-host plot arrangements, etc. For the modeling process, all the on-farm present cherry cultivars were categorized into four phenological groups: early, medium, late and very late, and for each group a representative cultivar was assigned from among those locally present on-farm. For each grid sector containing sweet cherry trees, appropriate representative cultivar was assigned according to the overall cultivar composition on the plot. Estimation of the initial pest population in each farm sector (expected adult emergence from the soil in spring) was made based on historic pest monitoring records, cultivar category, tree size, distribution, and canopy status etc.

b. *Phenology of fruit development:* The key points in host phenology, such as flowering, onset of fruit susceptibility to *R. cerasi* infestation and fruit maturity/harvest time were recorded for the main on-farm present sweet cherry cultivars. The points were defined as follows: time of flowering—when 30% of flowers open, onset of fruit susceptibility—fruit color change from dark green to yellowish—green (30% of fruits), fruit maturity/harvest time—actual harvest dates. The fruit susceptibility period was deemed to last from the time of fruit color change till harvest.

c. *Pest monitoring:* At the onset of 2015 season, on each farm, 7–8 Rebel traps were set for pest monitoring. Position of the Rebel traps on each farm is shown on **Figure 1**. The traps were checked at various, ca. weekly, time intervals, following the usual management practice. During each trap check, all the flies trapped were removed and their numbers recorded. When males and females were not separated, 1:1 sex ratio was assumed (Ozdem and Kilincer, 2008), and half of the catch (to represent only females) was used for simulations.

d. *IPM treatments:* For each farm, records were maintained about fruit development, fruit infestation at harvest, average weight of a single ripened fruit, approximate crop yield, pest management actions such as pest control sprays, type and dose of pesticide, time and area of application, etc. To assess fruit infestation, 100 fruits were randomly collected at the time of harvest and dissected. Crop yield was based on farm records, or on grower's assessment. Average weight of a single fruit was estimated by weighting ca. 100 mature fruits, randomly collected at harvest.

e. *Weather data:* For each farm, records of daily maximum, average and minimum temperatures (recorded at 2 m above the ground), rain, wind speed (at mid-day), and sunshine (radiation) intensity were used.

Statistical Analysis

The experimental, on-farm collected data were compared with the model-generated results of analogous "virtual" experiments. Simulation of each "virtual" experiment was replicated 5 times. Before comparisons, homogeneity of the model-generated results was verified using the chi square χ^2 test, and in all cases,

confirmed. The overall patterns of the experimental trap catches, both for the mark recapture experiment and on-farm pest monitoring, were compared with the model generated results using the chi square χ^2 test of goodness-of-fit. Due to small numbers of events, Monte Carlo simulations to corrected *p*-value from chi-square test was used. All the statistical analysis were carried out using R software version 3.1.3 (R Development Core Team, 2015). In addition, a simplified process control test was used, and it was assumed that the process is acceptably controlled (simulated), if the experimental points fall within the 3-sigma control limits for the respective simulated points, and relative distribution of the experimental and simulated points approximates random - no sequences of "+ + +" or "− − −" longer than 6 ($p < 1\%$).

RESULTS

Identification of the Biology Aspects Relevant to *R. cerasi*, and Model Adaptation

The aspects relevant to *R. cerasi* biology and model adaptation were identified based on literature review, the author's experience, and our auxiliary experiments and observations. The result of the review, the aspects selected for modeling, and their relevance to the model adaptation are outlined below, while the derived model processes and parameters were summarized in **Table 1**.

General Traits

The European cherry fruit fly is a univoltine pest, and the females, which emerge from the soil in spring, reach maturity within 5–14 days, and deposit eggs into cherry fruits causing crop damage (Łęski, 1963; Daniel and Grunder, 2012). Under typical field conditions, sex ratio of the adults emerging in spring approximates 1:1, and the majority of females are fertilized (Daniel and Grunder, 2012). Therefore, only females of *R. cerasi* were considered in the simulations.

The process of adult emergence is not synchronized, typically, it lasts 35–50 days, with a culmination in the middle of this period, when 70–90% of individuals emerge during the peak 14 days Vogt et al. (2010). Accordingly, such staggered emergence was modeled, with parameters adjusted to the local farm conditions. Effectively, this phenomenon generates 35–50 consecutive and overlapping age-sub-cohorts, which were modeled separately.

The overall mortality per generation, from the egg till the adult stage emerging from the soil next spring, fluctuates seasonally, usually within the range of 85–98% (Łęski, 1963; Vogt et al., 2010; Daniel and Grunder, 2012). Female longevity and fecundity varies among locations and seasons, but under the optimal conditions of unlimited food and fruit supply and the absence of any extrinsic mortality causes, the adult lifespan fluctuates around 60 days, with its maximum exceeding 90 days, while gross fecundity may reach 365 eggs/female, with average daily fecundity rates ranging from 0 to 10 eggs/female, according to female age (Köppler et al., 2008; Moraiti et al., 2012). Model parameters were adjusted accordingly, tuned to fit the published data for JKI strain (Köppler et al., 2008). In the absence of specific information, parameters of the JKI strain were deemed to approximate that of the other strains simulated in the reported study (BOKU and PC-Fruit).

Insect Mobility

The European cherry fruit fly is known for its intimate association with the host tree and its overall mobility largely restricted to the local canopy and its close neighborhood (Böckmann et al., 2012, 2014; Daniel and Grunder, 2012; Daniel and Baker, 2013). Based on our preliminary on-farm observations, the average area covered during a single local exploration errand was estimated at 100 sqm, which was used to determine the basic sector size (10×10 m) for all the arrays representing various aspects of the farm, and consequently—the spatial resolution of the whole simulation process. Earlier findings (Wiesmann, 1935; Łęski, 1963; Boller, 1969), and results of mark-recapture experiments conducted at JKI (Vogt et al., 2010) indicate that occasional micro-migration might exceed a few hundred meters. This was confirmed by our mark-recapture experiment, reported below, which revealed also substantial age-dependant differentiation in several aspects of female mobility, such as varied propensity to undertake the local exploration or on-farm movements, divergent spatiotemporal patterns of the on-farm dispersion etc. These results were taken into account in the process of adjusting the algorithms and parameters of the "virtual" insect mobility module. The possibility of occasional, temporal or permanent, out-of-the-farm migration of some individuals and/or arrival of a number of newcomers from the neighborhood—were also taken into account. Since the experimental farms were located within a similar landscape, a balanced scenario was assumed (in/out migration = 1). Furthermore, insect mobility was assumed to be modulated by the local niche conditions—be enhanced by decreased site attractiveness and/or excessive local pest density or fruit infestation.

Tree Canopy

Tree canopy constitutes the primary environment for the adult stages of *R. cerasi*, providing shelter and the key attributes required for reproduction. Frugivorous fruit flies, including *R. cerasi,* are known to respond from a distance to visual signals and tree canopy, and adjust their within-canopy behavior according to its size and structure (Prokopy, 1968; Boller, 1969; Katsoyannos et al., 1986; Prokopy et al., 1987; Stadler and Schoni, 1991; Senger et al., 2009; Daniel and Grunder, 2012). Chemical cues, emanating from canopy of various host and non-host trees also play, usually attractive, roles (Lux unpublished). Our on-farm observations indicated further, that apart from the individual tree size, aspects of canopy macro-structure, such as uniformity of tree distribution (tree blocks vs. patchy or scattered) or training the canopy into regular rows, row direction and depth in relation to the open transects, etc.—all play a role in shaping patterns of insect relocations. Consequently, such moderating effects were incorporated into the model.

Fruit Development and Suitability

The European cherry fruit fly is an oligophagous pest, and the presence of cherry fruits at the right stage of development is prerequisite to oviposition. Thus, the "basic" attractiveness of fruitless host tree canopy was assumed to fluctuate at moderate level (ca. 30% of the respective maximum) and substantially increase when the fruit suitable for oviposition becomes present. Details of the fruit development and concomitant physio-chemical changes, especially during its intense growth and early maturation stages, are still little understood (McAtee et al., 2013), and even less so, the corresponding evolution of the fruit sensory qualities and its attractiveness to the insect. Typically, the oviposition starts at mid-late stages of fruit growth, marked by color change from dark green to yellowish-green, culminates at late-growth or early fruit maturation stages and gradually declines until ripening or harvest (Vogt et al., 2010; Daniel and Grunder, 2012). The cultivar-specific periods of fruit suitability were established based on our on-farm records made for the selected cherry cultivars (**Table 2**). In the absence of specific data, the fruit suitability periods were estimated at 31 or 44% of the average flowering-to-harvest period typical for the cultivar, early or late, respectively, calculated backwards from the harvest time. The latter was based on our results (**Table 2**) and findings of Schumann et al. (2014) about seasonal dynamics of fruit development, in particular—relation between rapid acceleration in the increase of fruit mass and hue change.

Conceivably, various cherry cultivars may differ in their overall attractiveness and capacity to stimulate oviposition, and their suitability as hosts—capacity to support successful egg-to-larva development. For example, production of hard tissue secluding the eggs and thus reducing their development was reported for some cherry cultivars, such as Schattenmorelle (Thiem, 1954). Although, the model has in-built provision to cater for such phenomena, in the absence of relevant information, the peak attractiveness and the overall host-suitability were assumed equal for all the cultivars. It was assumed further that female intrinsic oviposition propensity be modulated by the status of the sector of her actual presence—enhanced by increase in the overall attractiveness of the local niche and abundance of suitable fruit, and decreased by excessive density of the local pest population or niche exploitation (fruit infestation).

Fruit Infestation

The European cherry fruit fly is known to utilize epideictic pheromone, deposited on the fruit by the egg-laying female immediately after oviposition to prevent repeated utilization of the same fruit and thus reduce the risk of intra-specific larval competition (Katsoyannos, 1975). This mechanism does not prevent occasional multiple fruit infestations at higher pest pressure, but because in most of the emulated scenarios the fruit infestation was much below 100%, it was assumed that all the eggs were always laid singly (1 egg/fruit). The "in-the-fruit" development time varies depending on temperature and fruit stage (Łęski, 1963; Vogt et al., 2010; Daniel and Grunder, 2012), and for the locations under the study was assumed to last 20–23 days. Based on our preliminary observations, to enhance model realism, a 5-day post-infestation fruit recovery time, counted from the day of the egg deposition, was assumed for the instances when an egg or young larva died soon after (e.g., due to application of a systemic pesticide), and the initial fruit injury healed without discernible or disqualifying post-infestation symptoms.

Fruit Harvest

In the absence of systemic pesticide application, harvest is a major factor reducing in-fruit-residing immature population and its carry-over to the next season (Daniel and Grunder, 2012). Therefore, harvest accuracy was estimated for each plot, and the

TABLE 2 | On-farm (PC-Fruit) recorded "Fruit suitability windows" for *R. cerasi* oviposition in various sweet cherry cultivars*.

Sweet cherry cultivar	Average F–Y period (days)	Average FSW (Y–H period)		Average harvest date
		(days)	(% of the F–H period)	
IDENTIFIED SWEET CHERRY CULTIVARS				
Hertford	59	23	28%	7th July
Kordia	50	29	37%	9th July
Karina	62	19	23%	10th July
Grace Star	54	26	33%	10th July
Lapins	61	30	33%	14th July
Regina	53	40	43%	27the July
Sylvia	50	44	47%	28th July
Sweetheart	59	45	43%	29th July
UNKNOWN OLD SWEET CHERRY CULTIVARS				
U1	–	45	–	22nd July
U2	–	45	–	22nd July
U3	–	45	–	22nd July
U4	–	41	–	25th July

**FSW, fruit suitability window; F, flowering time; Y, fruit colour change from green to yellowish-green; H, harvest time.*

larvae which failed to complete their "in-the-fruit" development period by the harvest time, were considered dead if the fruit was harvested, or assumed completing their development into a pupa if resident in a fruit left on the tree after harvest. Furthermore, because the harvested sweet cherries are typically consumed or processed without delay, additional 4-day period of "concealed" injury was assumed for the instances of just pre-harvest infestation, when *de facto* infested fruit still appears unblemished to the consumer. In all such cases, the eggs were counted toward the overall fecundity, but not to the next generation, and the fruits were treated as "un-infested".

Natural Enemies

Based on the analysis of historic data, for all sweet cherry cultivars, the average extrinsic adult mortality risk, due to the on-farm resident natural enemies and pathogens was estimated at 3% daily. Although, seasonal fluctuation is very likely, due to lack of specific data, this effect was assumed constant throughout the season. In our survey, out of 195 individuals of *Psyttalia carinata* (formerly *P. ragoleticola*) recovered, all originated from the larvae collected from wild cherry trees abandoned at WULS campus (34.39% parasitation rate), and none from the cultivated, unprotected cherries. Thus, the impact of larval parasitoids on the immature stages was assumed negligible, even in the absence of pesticide treatments.

Rebel Traps

Our experience suggests that the number of insects caught by a particular trap depends not only on the general population density, but also on the specific properties of the spot where the trap is located. Individual exposure to the trapping risk is not uniform on-farm, and strictly depends on its position relative to the trap, thus a patchy pattern of the trapping risk was assumed, mirroring trap distribution. The effective trapping range was estimated at 100 sqm, and only the insects present in such area were deemed exposed (in a stochastic sense) to the trapping risk. Implementation of such mechanism ensures that the phenomenon of gradual catch reduction, caused by temporary out-trapping the locally resident flies, was also emulated. Although modulation of the trap attractiveness, caused by seasonal changes in background canopy hue appears likely, due to lack of relevant data—no such effect was included. Based on the results of our mark-recapture experiment, variation in female responsiveness to the trap was assumed, from 80% after emergence, 100% at the peak of reproductive activity, and gradual decline to ca. 40% when 4–6 weeks-old. Because glue-covered traps, exposed in the field for extended periods (ca. 2 months in our study), gradually lose trapping efficiency due to build-up of dust, debris and non-target organisms, for a new Rebel trap, the initial daily trapping risk was estimated at 5%, with a 1% daily decline.

Pesticide Application

The insects present in or entering pesticide application zone were deemed exposed to additional mortality risks. The date and area of each application were taken into account, along with the estimated temporal profiles of its residues (Lazić et al., 2014) translated into mortality rates of adult flies, and whenever relevant, immature stages developing in the fruit. In such the areas, mortality risk due to the local natural enemies was temporarily reduced to reflect patterns of the pesticide-imposed transient suppression. Specific border effects on the pace and pattern of predator recovery, relative to the size and shape of the area of pesticide application, were also taken into account.

Role of Weather Conditions

The local weather conditions determine behavior of *R. cerasi* and, at more extreme spells, impose mortality risks. The flies are active during warm, calm and sunny days, with temperature above 15°C required for mating (Katsoyannos, 1979; Daniel and Grunder, 2012), and above 16°C for oviposition (Boller, 1966; Daniel and Grunder, 2012). Our observations also confirmed that fly mobility and explorative activity is reduced during cloudy days, especially with some rain or wind. Bad weather spells, with severe rains, especially when combined with strong winds, were assumed to increase fly mortality, according to weather severity and sheltering capacity of local tree canopy.

Model Validation

The validation process consisted of two major steps, simulation of (1) the mark-recapture experiment to validate the model's "insect mobility module" and (2) the IPM experiments conducted on two sweet cherry farms (one in Austria and one in Belgium) to validate the "virtual farm" concept.

Simulation of the Mark-Recapture Experiment

The "insect mobility module" is the key model component, which determines on-farm movements of the "virtual" insects. It was evaluated against the mark-recapture experiment, conducted on JKI farm (shown on **Figure 1**), located in Dossenheim, Germany. The model was set to emulate distribution and the recapture process for each of the three female age categories (5-, 14-, and 28-days-old). The model-generated results were compared with that obtained on-farm. Regardless of the age category, the flies released into the open field remained alive and could be re-trapped during at least 2 weeks post release. In general, out of over 1000 individually marked flies, ca. 6% (64 flies) were trapped. Out of over 300 young (5-days-old) females released—30 individuals (8.7%) were re-trapped, most of them in the release zone (83%), and the remaining 10 and 7% in the zones 2 and 3, respectively. Mature females (14-days-old) were re-trapped at nearly the same rate (8.8%), but relatively fewer in the release zone (55%) and more in the zones 2, 3, and 4 (17%, 10%, 17%, respectively). For the old females (28-days-old), the overall re-capture rate was much lower (1.6%), and thus the catches were much more erratic. Nevertheless, almost half of the re-trapped females were caught in the distant zones (3 & 4), and interestingly, none in the adjacent one (zone 2).

The model-generated results of the "virtual" mark-recapture experiment largely mirrored that obtained on-farm, both in terms of their spatial and temporal patterns (**Figure 2**). For each of the three female age categories, no significant discrepancy between the respective simulated and experimental results was detected (**Table 3**). Furthermore, barring the exception of one

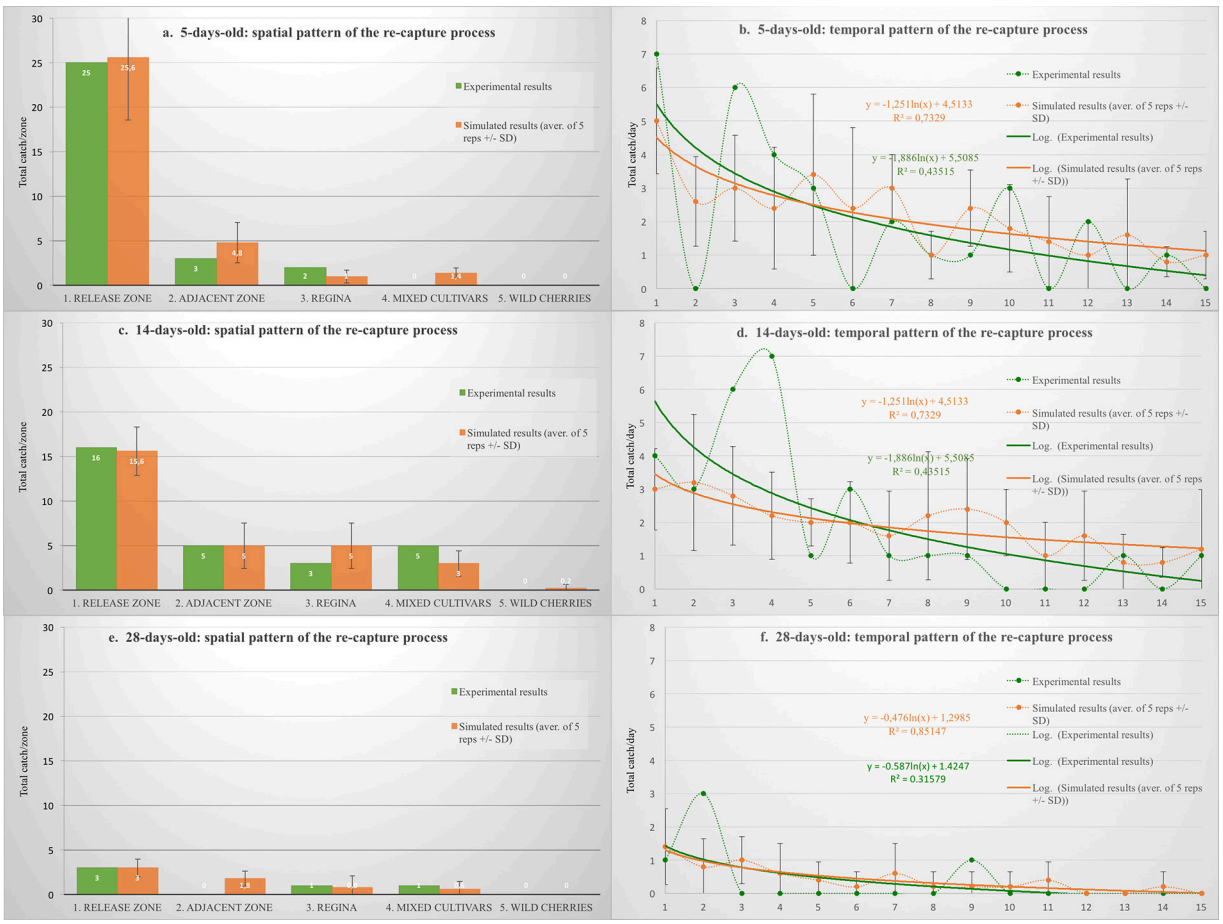

FIGURE 2 | The mark-recapture experiment: comparison between experimental vs. model-generated age-dependent recapture patterns.

point only (**Figure 2D**, day 4), all other experimental points fell within the 3-sigma control limits of the respective simulated data points, and the relative deviations of the simulated and experimental points did not depart from random (no sequences of "+ + +" or "− − −" longer than six).

Admittedly, due to laborious nature of the mark-recapture experiment, the numbers of the experimental data points were low, which prevented establishment of precise "reference" distribution patterns, and thus more rigorous model calibration. Provision of over 1000 individually marked flies, ready for release the same day and representing the three, broadly different age cohorts, presented a challenge. Several thousand of difficult to obtain *R. cerasi* pupae had to be used for this purpose, and substantial increase of these numbers was not feasible. Nonetheless, the results revealed earlier unknown, age-related differences in *R. cerasi* distribution propensities, distances and patterns, which were broadly replicated by the model.

Simulation of the On-Farm IPM Experiments

The IPM experiments were conducted on BOKU farm located near Vienna in Austria and PC-Fruit farm located in Metsterenweg in Belgium. Each farm contained a number of plots with sweet cherry cultivars of varying phenology, some old abandoned cherry trees, plots with non-host fruits trees, wild trees and "empty" plots with perennial or non-tree crops. The two farms differed in their spatial arrangement, size and age of the host and non-host trees (**Figure 1**).

For both farms, the 15th of May was set as the first day for all simulations. However, the time of the fly emergence and the onset of the cherry season varied between the two locations by ca. 14 days, according to their latitudinal difference, and alike, the phenological type and composition of the main cherry cultivars. Thus, for the BOKU farm, Burlat, Blaze Star, Kordia, and Regina were used to represent the main four phenological groups. Burlat was harvested on 4th June, Blaze Star on 18th June, Kordia partially on 25th June and later on 2nd July, Regina on 2nd and 9th July. On the PC-Fruit farm, only medium and late fruiting cultivars were grown, the latter included also some "old" unidentified sweet cherry cultivars. Accordingly, Kordia, Lapins, Regina, and Sweetheart were used as the main phenological representatives. Kordia was harvested on 9th July, Lapins on 18th July, Regina on 25th July, and Sweetheart on 29th July.

On both farms, natural, and well-established *R. cerasi* populations were present, but their overall densities and temporal

TABLE 3 | Comparison between re-capture patterns: experimental vs. model-generated.

Source data	Chi-square	df	*p*-value
Figure 2A. 5-day-old: spatial pattern	2.0350	4	0.5681
Figure 2B. 5-day-old: temporal pattern	12.0896	14	0.5991
Figure 2C. 14-day-old: spatial pattern	1.2044	4	0.7964
Figure 2D. 14-day-old: temporal pattern	10.9554	14	0.7567
Figure 2E. 28-day-old: spatial pattern	1.8145	3	0.6118
Figure 2F. 28-day-old: temporal pattern	5.6095	11	0.8961

patterns varied substantially. With the same number (8) of the Rebel traps set on each farm, during the season, 953 and 123 females were caught, and the maximum catch (on control plots) was recorded on 11th June and on 16th July, on BOKU and PC-Fruit farm, respectively. On both farms, *R. cerasi* was controlled on one plot only, and no other IPM treatments against the pest were made on the farm remainder. In BOKU, a pesticide was applied only once, on the plot (ca. 0.3 ha) with trap No 7, containing (almost exclusively) Kordia and Regina cultivars. The pesticide, Mospilan 20 SG (0.0375%), was cover-sprayed on 5th June, at the time of the fruit color change on Kordia (from green to yellowish-green). The same cultivars, Kordia and Regina, were present on the protected plot (ca. 0.35 ha) on the PC-Fruit farm. Part of the plot, containing traps No 6 & 7, was spot-sprayed with a mixture of a bait and pyrethroid, and the part containing the trap No 8 was cover-sprayed with Spinosad. The treatments were repeated twice, on 16th and 30th of June.

Spatial and temporal patterns of the trap catches during the pest monitoring conducted on each farm and the model-generated simulation results are presented on **Figures 3A–D**. In spite of substantial differences between the two farms, the simulated pest monitoring largely conformed to that obtained on-farm. No significant discrepancy between the overall patterns was detected (**Table 4**), and most of the data points passed the simplified process control test and fell within the respective 3-sigma control limits. However, failure to detect discrepancy in the overall patterns has to be interpreted cautiously, especially when some trap catches are inherently low and erratic, such as early and late in season, or in the traps located outside of the host zones. Indeed, in some of such cases, experimental results fell outside of the 3-sigma control limits (**Figure 3A** Trap 5; **Figure 3C** catches on: 19.05 & 2.07; **Figure 3D** catches on 4.06).

Comparison of the experimental and simulated fruit infestation patterns, for each of the four representative cultivars at their respective harvest times, is presented on **Figures 3E,F**. Simulation of fruit infestation is prone to errors. While numbers of eggs laid in each plot or farm sector are emulated with fair accuracy, their translation into the fruit infestation depends on the precision of the local fruit load (productivity) estimation. Experimental assessment of the actual fruit productivity and infestation is laborious, therefore the calculation was based on quite broad estimations, generated by the model, and compared with a limited number of experimental records. Nevertheless, both for the control and the pesticide-treated plots, and regardless of cultivar phenology and harvest time, the simulated results of fruit infestation conformed acceptably to the experimental data. No significant discrepancy between the overall patterns was detected (**Table 4**), and only a few data points, all with low and thus erratic infestation, fell outside the respective 3-sigma control limits (**Figure 3E** Burlat 4th June and Kordia 25th June; **Figure 3F** U4).

Admittedly, the appearance of some data points outside the 3-sigma control limits indicates likely imperfections of the simulation process. On the other hand, it is worth to emphasize that the detected discrepancies were numerically small, related to the inherently erratic data, thus were of very limited practical importance. The overall similarity of the simulated and experimental results corroborates, that the model emulated the key on-farm processes, such as phenology of various host cultivars, patterns of pest emergence and mortalities, its on-farm movements and fruit infestation, and also IPM actions—pest monitoring (for specific trap locations) and effects of the local pesticide application. The results indicate that the model-based "virtual farms" constitute acceptable representation of the respective real farms, where the IPM experiments were conducted.

DISCUSSION

Agent-based models permit incorporation of a large number of component processes, which determine behavior of their agents, or just modify it under certain circumstances. Consequently, model adaptation constitutes critical, but also an open-ended process, mirroring the current status and progress in our understanding of the causative agent (here: *R. cerasi*). The choice of the processes to be modeled, and the quality of the input information—jointly determine the relevance and precision of the model. Explicably, in simulation of complex systems, rigorous evaluation of all assumed relations and parameters may not be feasible. The purist approach—incorporating into the model only rigorously established and parametrized processes, although tempting and warranting formal methodological correctness, also comes at a price. Discounting plausible, but superficially quantified aspects of biology *de facto* entails adopting "hidden," and frequently much less correct, default patterns for the "discarded" processes—a zero-order linear relations.

Being faced with such dilemma, we have chosen a pragmatic approach—including into the model also putative, and in some cases, only tentatively parametrized processes, in order to construct a "frame-model" capable to provide initially-acceptable emulation of the system. Indeed, the experience shows, that viable agent-based models, generating plausible answers to complex questions, can be constructed even in the shortage of detailed knowledge about the system (An et al., 2009). Later on, with new information, the rules and parameters can be fine-tuned, without having to modify the entire model.

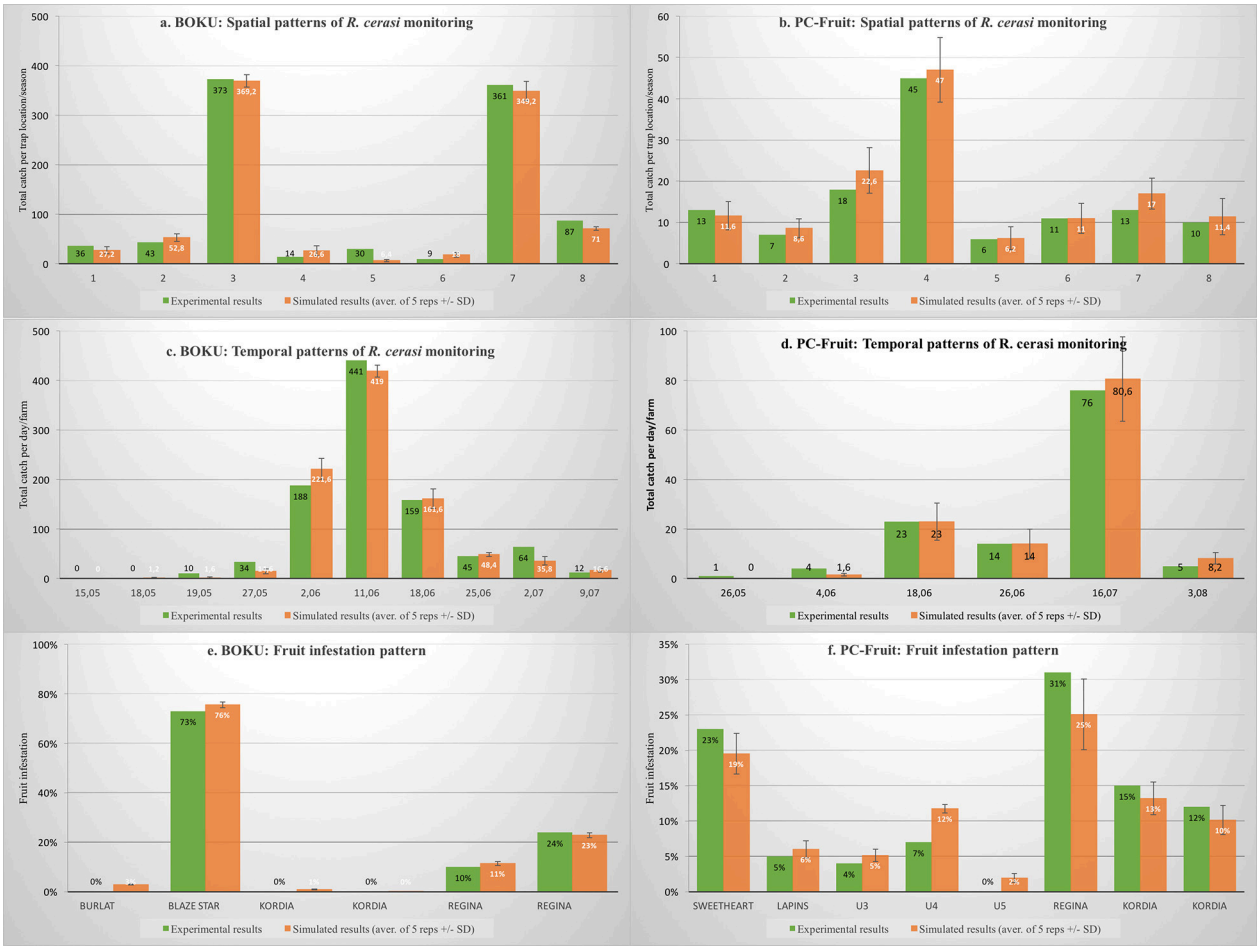

FIGURE 3 | On-farm IPM experiments: comparison between experimental vs. model-generated spatial and temporal pest monitoring and fruit infestation patterns.

Accordingly, a simple approach to model validation was taken—testing whether the model can reproduce empirical data with reasonable accuracy. The results, presented above, confirmed the general capacity of the model to emulate the key on-farm processes and satisfactorily reproduce IPM experiments conducted on the respective farms. But ultimately, the model value rests in its ability to extrapolate to situations beyond those originally observed on-farm, to provide new insights extending beyond what was already known. Results of such applications of the model are discussed below.

Insights into the Seasonal Patterns of Pest Population Density

Seasonal patterns of *R. cerasi* population density, simulated under assumption of NO pesticide application on the whole farm, are presented on **Figure 4**. In BOKU, the simulated peak of population density occurred during 20th–22nd season day (3rd–5th June), although a sizeable population continued until 50th day (on late cultivars). In PC-Fruit, the population culminated around 35th day and, due to more extended and "flat" profile of pest emergence from the soil in spring, continued at such high level until ca. 45th day, afterwards gradually declined, but still a sizeable population continued beyond 75th day.

TABLE 4 | Comparison between pest monitoring and fruit infestation patterns: experimental vs. model-generated.

Source data	Chi-square	df	*p*-value
Figure 3A. BOKU: spatial pattern of pest monitoring	1.364	7	0.2582
Figure 3C. BOKU: temporal pattern of pest monitoring	2.541	9	0.1124
Figure 3B. PC-Fruit: spatial pattern of pest monitoring	3.011	7	0.4721
Figure 3D. PC-Fruit: temporal pattern of pest monitoring	0.875	5	0.8541
Figure 3E. BOKU: fruit infestation pattern	1.718	5	0.4217
Figure 3F. PC-Fruit: fruit infestation pattern	2.321	7	0.3547

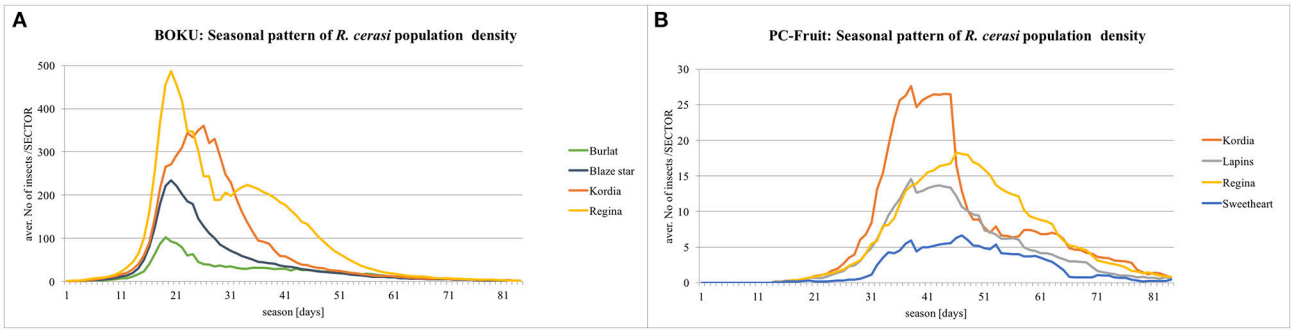

FIGURE 4 | Simulated seasonal patterns of *R. cerasi* population density. Assumption: NO pesticide treatment on the whole farm.

The difference between the farms in the simulated and on-farm recorded profiles of fly emergence was probably caused by more diversified topography and ground cover of the PC-Fruit farm, and generally lower temperatures prevailing during the pest emergence—from 5th till 40th season day (19th May–23rd June) the average daily temperature was 3°C lower (22.1 and 19.1°C, BOKU and PC-Fruit, respectively). Furthermore, in PC-Fruit only medium and late maturing cultivars are grown, characterized by occurrence of late and prolonged "fruit suitability windows" (**Table 2**), hence a degree of the local pest adaptation (delayed and diffused emergence to cover delayed and longer fruit suitability)—appears likely.

In general, the European cherry fruit fly is a "sedentary" pest, closely linked to its host tree (especially when isolated), with relatively limited propensity for distant translocations (Daniel and Grunder, 2012). However, within the plots with continuous canopy coverage, local explorations and transfers "from tree to tree" are common (Wiesmann, 1935; Łęski, 1963; Daniel and Wyss, 2009). Thus, when two or more cultivars, substantially different in their phenology and the extent of "fruit suitability windows," are present on the same plot, local shifts "within-the-plot" are likely. Indeed, on the simulated patterns, both for BOKU and PC-Fruit farm, on the plots containing Kordia and Regina cultivars, a degree of a local shift of the pest is visible (**Figure 4**), with the majority seen first on Regina, shifting later toward Kordia at the peak of its fruit suitability. When Kordia fully ripened and the fruit became dark (nearly black) and thus less attractive, the flies shifted back to the later ripening Regina, having still suitable and attractively red fruit.

Transfers among the plots within the farm, although occur and become progressively more frequent with advancing female age and decreasing availability of suitable fruit, are generally limited. In consequence, substantial differences can be maintained in the density of the resident pest populations among the isolated plots containing various cherry cultivars. The local density is relative to the capacity of the cultivar to sustain complete in-fruit immature development cycle, which is much higher for later maturing cultivars with more extended "fruit suitability window," better correlated with the period of the pest's peak fecundity.

All these phenomena are well reflected by the simulated results, but importantly, the model allows to assess their local magnitude and implications for various IPM scenarios.

Insights into the Impact of Cultivar Phenology on the Effective Female Fecundity

The potential lifetime fecundity (365 eggs/female) adopted for model calibration, based on the laboratory data obtained under optimal conditions, unlimited food and fruit supply and the absence of any extrinsic mortality causes (Köppler et al., 2008; Moraiti et al., 2012), may appear inconsistent with the reports about the on-farm estimated effective fecundity (30–200 eggs/female; Łęski, 1963; Daniel and Grunder, 2012). However, alike on a real farm, the "virtual" females are also exposed to a daily changing configurations of various mortality risks, are challenged by the necessity to survive 5–10 days to reach maturity and attain the capacity for egg-laying, are also subject to individual aging and resultant changes in fecundity, and often, faced with imperfect alignment between the periods of their peak fecundity and the local fruit "suitability windows." Thus, the simulated effective average fecundity of a "virtual" female newly emerging from the soil in spring was strongly dependant on the phenology of the cultivars prevailing on the plot—and ranged from less than 1 on Burlat, ca. 6 on early cultivars up to ca. 27 eggs/female on the late ones. As expected, IPM treatments reduced lifespan on the females dwelling on the treated plots, and accordingly, their net fecundity, but the model allowed for approximate quantification of such effects according to the local conditions (**Table 5**).

Insights into Seasonal Modulation of Pest Age Structure

The **Figure 5** shows the timing and cumulative density (for the whole farm) of the main four female age categories; immature (1–10 day-old), mature (11–30 days-old), old (31–60 days-old) and senile (over 60 days-old), simulated under the assumption of NO pesticide application on the whole farm. The overall seasonal patterns of female age structure differ between the two farms. In BOKU, fairly clear succession of the age cohorts can be seen, in

TABLE 5 | Influence of host phenology and IPM treatments on the effective net fecundity of *R. cerasi* females.

Net effective fecundity/female emerging from the soil in spring*									
BOKU					PC-Fruit				
Sweet cherry cultivar	NO pesticide[A]		Pesticide treatment[B]**		Sweet cherry cultivar	NO pesticide[A]		Pesticide treatment[A]**	
	Aver.	SD	Aver.	SD		Aver.	SD	Aver.	SD
Burlat	0.69[c]	0.06	0.56[c]	0.02	Lapins	12.25[c]	0.65	11.90[b]	0.18
Blaze star	6.19[b]	0.05	5.51[b]	0.07	Sweetheart	25.50[a]	2.29	25.39[a]	1.19
Kordia	24.90[a]	0.16	**8.60**[a]	0.08	Kordia	7.68[d]	0.15	**5.28**[c]	0.39
Regina	26.89[a]	0.15	**9.84**[a]	0.18	Regina	20.90[b]	0.38	**14.47**[b]	0.20

*Different capital letters indicate significant differences between respective NO pesticide and Pesticide treatment; different small letters indicate significant differences between cultivars.
**Pesticide treatments are indicated by bold letters.

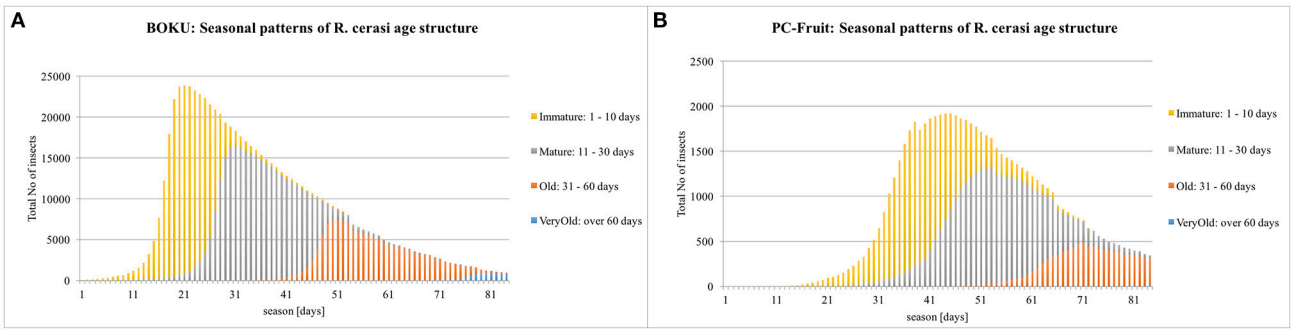

FIGURE 5 | Simulated seasonal changes in *R. cerasi* age structure. Assumption: NO pesticide treatment on the whole farm.

contrast to extensive overlap and largely concurrent presence in PC-Fruit.

The four female age categories differ in their behavior, fecundity, and thus importance from the "farmer's point of view." Immature females, although present on farm and trapped during monitoring, are unable to infest the fruit and thus do not pose any threat to the crop. At this stage, their exploratory mobility is reduced. Maturing females increase their propensity for local explorations, and soon after, attain the peak of their reproductive capacity. This stage is the most damaging and thus should become the primary target of any IPM. Old females still have substantial fecundity and thus crop damaging potential, and enhanced propensity for longer explorative errands. However, after over 30 days of exposure to numerous mortality risks, their population is already decimated, and therefore, of lesser practical importance. The oldest category, over 60-days-old, although retaining some "residual" fertility, due to very low densities and probably reduced responsiveness to Rebel traps, are barely visible on farm and their practical impact is negligible.

The simulation indicates, the main IPM effort shall be focused from ca. 20th till 45th season day in BOKU, and for much longer period, from ca. 30th till 75th season day, in PC-Fruit.

Insights into Mechanisms of the Local IPM

The IPM regime applied in BOKU—a single cover-spray with a systemic pesticide, Mospilan (acetamiprid)—targeted primarily the immature stages developing in the fruit, though the pesticide has also short-term knockdown action against the adults. The pesticide was applied at the time of fruit-color-change in Kordia. Although the treatment ensured weeks-long systemic action, effectively killing the immature stages developing in the fruit, its efficacy gradually decreased before the harvest. Nevertheless, the protection was effective for Kordia, but the residual pesticide activity was insufficient to prevent substantial infestation of the later ripening Regina. Simulations of the on-farm experiment reveal, that if the daily larval mortality rate in the fruit (caused by the systemic pesticide) drops below ca. 60% during a few days (4–8) before the harvest, substantial crop damage is unavoidable. When a complete removal of this residual protection was simulated for the last 7 days, the infestation of Regina jumped to ca. 68%, compared to 24% recorded on the farm. The results explain why, with the IPM relying on the use of systemic pesticides and targeting the immature stages of the pest, it is virtually impossible to produce a "maggot-free" and truly "pesticide-free" fruit at the same time. This difficulty was recognized by the growers, who prompted EFSA to re-evaluate the formal EC MRL (Maximum Residue Level) for acetamiprid residues in sweet cherries, and increase the threshold from the earlier 0.2 mg/kg to 0.5 mg/kg (EFSA, 2010).

On both farms, the adult populations were substantially reduced by IPM programmes (single spray in BOKU and 2 treatments in PC-Fruit, both on the plots containing Kordia and

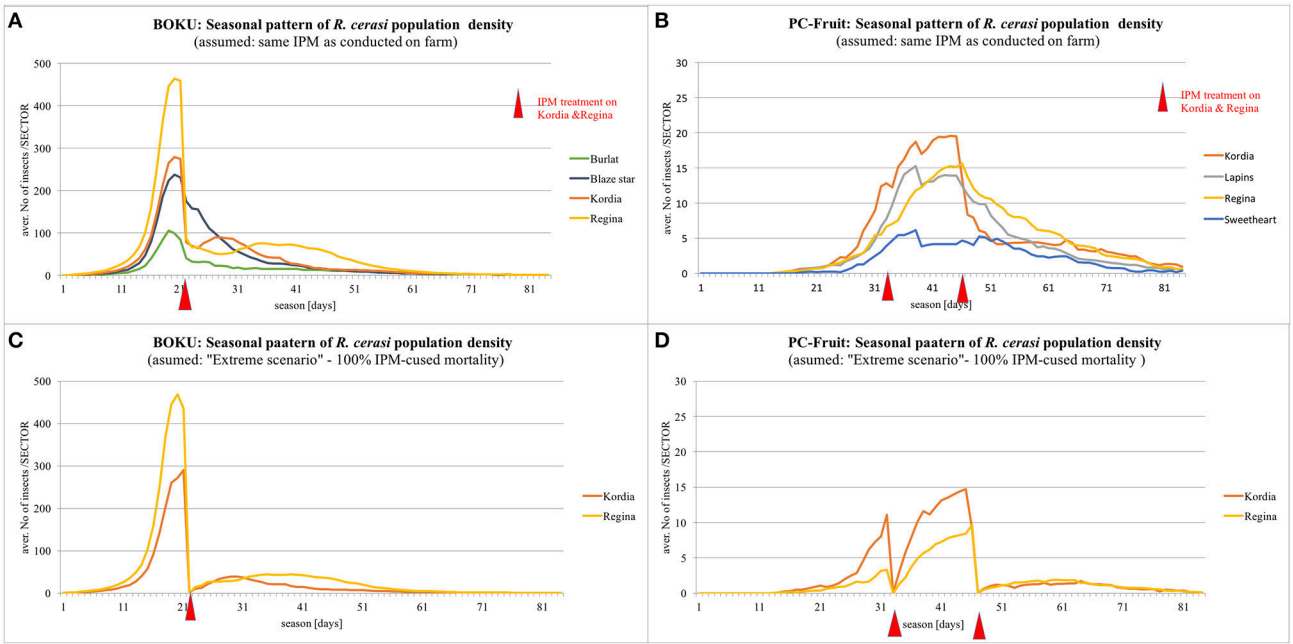

FIGURE 6 | Reduction and resurgence of *R. cerasi* population after IPM treatments.

Regina cultivars; **Figures 6A,B**) compared to the "NO-treatment" scenario (**Figures 4A,B**). However, they failed to eliminate the flies entirely, and prevent crop infestation. Even when a more extreme scenario was simulated—assuming complete absence of the pest on all other cherry plots except the ones treated with a pesticide, and on the latter, application of a short-acting (1 day only), knockdown, non-systemic pesticide inflicting 100% mortality to the adults present on the plot, applied in the same regime (timing and repetitions) as the experimental IPM treatment—still post-treatment pest populations re-appeared on the treated plots (**Figures 6C,D**) and caused crop damage (ca. 40–50% of that recorded the on-farm), both in the case of BOKU and PC-Fruit.

The source of the post-treatment population is not immediately clear, it may originate either from active immigration of females from the nearby plots, and/or "from the local soil"—through late emergence of the flies still present on the plot. To assess the relative contribution of the two "resurgence" pathways, several scenarios were simulated for each of the two farms, assuming various combinations of the presence or absence of flies on the treated and/or on all other (NON-treated) plots. The IPM treatment, if deemed applied, was always the same as the experimental treatments applied during the field experiments on the respective farms. The results, presented in **Table 6**, reveal that both in BOKU and PC-Fruit case, the major source of the post-treatment population increase was present on the treated plot—the late flies emerging from the soil after the treatment. The impact of this phenomenon on the effectiveness of IPM was more acute on the PC-Fruit farm, due to delayed and more prolonged fly emergence process. Relative contribution of the flies immigrating from the nearby plots, although considerable, was of lesser importance, and was dependent on the local farm configuration and distances to the nearby plots.

This phenomenon, frequently neglected or not fully realized, explains the challenges faced by the IPM programmes targeting only the adult flies, and reveals the importance of taking into account the local farm specificity in designing IPM strategy.

Insights into the Carry-Over of Pest Population–the Next Season Outlook

Understanding the local carry-over mechanism of the pest population to the next season is of utmost importance for successful IPM. The European cherry fruit fly, being a univoltine insect, has no capacity to multiply its adult population during the season, thus the carry-over of the immature stages pupating in the soil entirely determines the level of the next season threat the pest can pose to the crop. Although, when mature, the larvae actively depart from the fruit and jump into the soil, it is widely believed that harvest is one of the primary mechanisms for mass-removal of larvae from the orchard and thus one of the main mortality factors (Boller, 1966; Daniel and Grunder, 2012). Consequently, complete harvest is recommended as a measure of pest management. However, the extra effort and cost to increase the harvest accuracy from the usual ca. 80% to the required 100% is prohibitively high, which is the reason for low adoption of this measure by fruit growers.

To visualize the impact of harvest completeness on the carry-over of fly population in relation to phenology of the on-farm present sweet cherry cultivars, two scenarios were simulated for each of the farms: assuming either 80 or 100% harvest accuracy,

TABLE 6 | Contribution of various pest sources to the post-treatment population resurgence.

Assumed scenario				Fruit infestation (Regina cultivar)			
Scenario ID	Pest source		IPM treatment	BOKU		PC-Fruit	
	The treated plot	NON-treated plots		Aver. (%)	*SD* (%)	Aver. (%)	*SD*
a	x	x	x	22.84	0.87	25.08	4.49%
b	–	x	X	5.91	0.88	3.37	0.99%
c	X	–	X	17.87	0.84	28.24	3.55%
d	x	x	–	43.01	1.68	49.30	7.02%

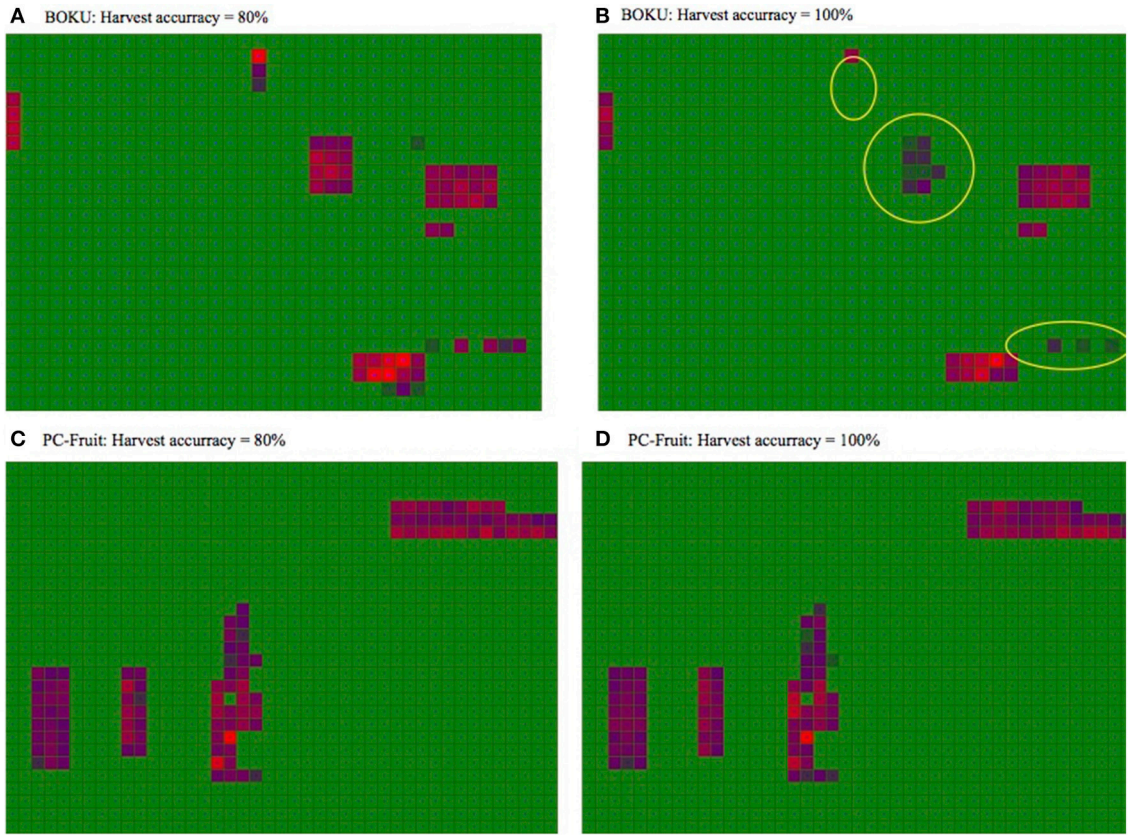

FIGURE 7 | Impact of harvest accuracy on the carry-over of pest population and anticipated emergence next spring. On the diagrams, intensity of the red hue corresponds to the local density of overwintering pupae.

with the harvest conducted at the same time and with the same IPM regime as that applied during the on-farm experiments. The results, presented on **Figure 7**, show that the impact of the complete harvest is substantial and clearly visible (yellow frames) only in the case of the medium-maturing cultivars, present in BOKU. But even on this farm, the difference for late varieties is negligible. On PC-Fruit, where late cultivars were grown, most of the larvae completed development and left the fruit before harvest, hence its completeness appears to have no practical bearing on the next year population.

Notwithstanding the potential of this measure when applied on early and medium maturing cultivars, in the light of our results, the universal value of the recommended harvest completeness appears questionable, especially in the areas where late cultivars are predominantly grown.

CONCLUDING REMARKS

The model represents bottom-up "ethological" approach to the site-specific IPM, focused on behavior of the individual insects—the primary actors determining local IPM performance. The results demonstrate that large amounts of quantified information about various aspects of the local farming system, pest biology and behavior can be consolidated and

embedded into the model, and converted into an operable site-specific IPM enhancement tool. Although, still imperfect, the model generates projections closely mirroring the results of the on-farm conducted IPM experiments. The model has in-built provisions to absorb new and more detailed pest-relevant and farm-specific information, and thus gradually improve its local relevance and robustness of its projections.

In its current state, it constitutes a viable "virtual" representation of the target sites and allows for "virtual" evaluation of numerous site-specific IPM scenarios. Admittedly, implementation of such tool will not eliminate field experiments, but can radically shorten the usual "development trajectory" by substituting major part of long-term and expensive on-farm experiments by their "virtual" emulations.

The "virtual insect" module was designed in a generic from, and can be adapted to a number of insect species of various biology. Its variants adapted to the target insect (pest) become strictly species specific, and can be used to drive any number of "virtual farm" sub-modules, where the same pest is the causative agent. The process of adaptation, both to the insect and to the specific farm, is largely "researcher driven." But once the farm is characterized and the model tuned to the local conditions, unlimited number of site-specific pest management scenarios can be modeled and evaluated. At this stage, the farmer can take the lead in formulation of various IPM scenarios, and make management decisions based on the received assessment of their efficacy and cost/benefit. To compare the effects of the chosen scenario with the results of its implementation on-farm, the farmer will have to provide information very similar to that normally collected, such as pest monitoring data, records about the IPM treatments, host tree phenology, fruit infestation and yield.

For each scenario, the simulation process generates substantial volume of information, normally not accessible during experimental work, which provides insights into the processes operating on the particular farm, such as seasonal patterns of pest emergence, density and age structure, seasonal patterns of pest mortality caused by aging, natural enemies, bad weather spells, pesticides, trapping etc., phenology for the main host cultivars, spatial patterns of insect translocations within the farm, emerging patterns of fruit infestation, anticipated patterns of female emergence next season, etc. A typical output, generated by the model after each simulation run, is presented in the Complementary materials.

A converse application of the model is also possible, for modification of the farm topography and development of pest-resilient landscape and site-specific IPM. The model also has the potential for conversion into a site-specific forecasting tool, once more detailed and complete information about the impact of climate on the host phenology and pest behavior becomes available.

AUTHOR CONTRIBUTIONS

SL conceived the project, contributed proprietary generic PESTonFARM model, made all model adaptations (conceptual modifications, algorithm and code writing, assumptions and calibrations), executed and interpreted all the simulations used in the manuscript. AW conducted on-farm surveys and detailed local inventories for all sites, and executed the on-farm observations and experiments on *R. cerasi* behavior and ecology. HV provided access to extensive data, including historic information about experiments conducted in the past at JKI on *R. cerasi* ecology, and supplied insect material (*R. cerasi* pupae) for all the experiments. TB, AS provided access to historic data about *R. cerasi* IPM and ecology relative to the PC-Fruit and BOKU sites, respectively, and conducted the local on-farm IPM experiments and observations on sweet cherry phenology. MS provided methodological comments and executed all statistical analyzes in discussion with the remaining authors. SL wrote the first draft of the manuscript, and all authors contributed to the final version.

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Population Dynamics and Flight Phenology Model of Codling Moth Differ between Commercial and Abandoned Apple Orchard Ecosystems

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Apple orchard management practices may affect development and phenology of arthropod pests, such as the codling moth (CM), *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), which is a serious internal fruit-feeding pest of apples worldwide. Estimating population dynamics and accurately predicting the timing of CM development and phenology events (for instance, adult flight, and egg-hatch) allows growers to understand and control local populations of CM. Studies were conducted to compare the CM flight phenology in commercial and abandoned apple orchard ecosystems using a logistic function model based on degree-days accumulation. The flight models for these orchards were derived from the cumulative percent moth capture using two types of commercially available CM lure baited traps. Models from both types of orchards were also compared to another model known as PETE (prediction extension timing estimator) that was developed in 1970s to predict life cycle events for many fruit pests including CM across different fruit growing regions of the United States. We found that the flight phenology of CM was significantly different in commercial and abandoned orchards. CM male flight patterns for first and second generations as predicted by the constrained and unconstrained PCM (Pennsylvania Codling Moth) models in commercial and abandoned orchards were different than the flight patterns predicted by the currently used CM model (i.e., PETE model). In commercial orchards, during the first and second generations, the PCM unconstrained model predicted delays in moth emergence compared to current model. In addition, the flight patterns of females were different between commercial and abandoned orchards. Such differences in CM flight phenology between commercial and abandoned orchard ecosystems suggest potential impact of orchard environment and crop management practices on CM biology.

Keywords: codling moth, phenology, flight model, commercial orchards, abandoned orchards, insecticide resistance

INTRODUCTION

Codling moth (CM), *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), is an economically important deciduous orchard pest in the northeastern (Dean, 1989) as well as in the western U.S. apple growing regions (Beers et al., 1993). In Pennsylvania, it has been a major pest in all apple growing regions (Hodgkiss et al., 1934; Worthley and Marston, 1935) for more than 70 years, and began to re-emerge as a serious pest in the late 1990's (Hull et al., 2001).

Pest management approaches including different insecticides (Hull et al., 2009a,b) and mating disruption products (Joshi et al., 2008; Bohnenblust et al., 2011a) are used for controlling both adults and larvae (such as CM and the oriental fruit moth [OFM], *Grapholita molesta* [Busck]) in commercial apple orchards in this region. At present, growers mainly time insecticide sprays against CM based on a phenology model (PETE - prediction extension timing estimator-henceforth referred as "PETE model") developed initially in Michigan in the 1970's (Riedl et al., 1976; Welch et al., 1978) using insecticide susceptible populations in non-commercial/abandoned apple orchards. Since then CM has developed resistance to various insecticides in commercial apple orchards around the world (Riedl et al., 1986; Bush et al., 1993; Varela et al., 1993; Knight et al., 1994; Dunley and Welter, 2000; Mota-Sanchez et al., 2008). In Pennsylvania apple orchards, widespread insecticide resistance has been observed in CM populations over the past 15–20 years (Krawczyk and Hull, 2005). These insecticide-resistant CM populations may develop at different growth rates as reported elsewhere (Lue, 2005), and it is very likely that CM phenological predictions are different from the currently used PETE model for many Pennsylvania apple orchards, and may be in other apple growing regions. Lue (2005) reported that insecticide-resistant CM populations may develop at different growth rates, and may have different flight emergence/spring emergence patterns than the normal (i.e., non-resistant) populations. Such differences may result in mistimed insecticide applications, reduced pesticide effectiveness, and pesticide overuse in commercial orchards due to the poor predictive performance of the model developed from non-resistant populations. Predictive models developed from a normal (insecticide susceptible) population of CM should include adjustments to accommodate life history changes, such as local ecotype evolution and pesticide resistance.

Population dynamics and seasonal phenology of insect pests (i.e., CM) is generally influenced by several biotic and abiotic factors. Insects are poikilotherms, as their body temperature is regulated by the ambient temperature. Effect of temperature among other factors on the development and seasonal phenology of various insect pest species (Messenger and Flitters, 1958; Allen, 1976; Akers and Nielsen, 1984; Wagner et al., 1984; Damos and Savopoulou-Soultani, 2008) including CM (Riedl et al., 1976; Welch et al., 1978; Jones et al., 2013; Kumar et al., 2015) had been widely studied in the past. Such findings have also been widely used in modeling phenology and predicting development or life cycle events of respective insect species over time (Damos et al., 2011), and have been applied extensively in integrated pest management (IPM) programs of various tree fruit pests (Riedl et al., 1976; Welch et al., 1978; Croft et al., 1980; Pruess, 1983; Knight, 2007; Damos, 2009; Damos and Savopoulou-Soultani, 2010). Although temperature is known as the major factor influencing CM development and phenology, other environmental factors such as the effects of crop management and orchard practices may also have influence on CM biological parameters, and their role cannot be underestimated.

Considering the potential impacts of orchard environment and pest management practices on the developmental biology and phenology of CM, this study was designed to address the following questions: (1) Is CM flight phenology in commercial and abandoned orchards different than those predicted by the currently used PETE model (developed in 1970s); (2) does the flight phenology of male and female CM differ in commercial and abandoned orchard ecosystems; and (3) how do the re-specified models perform in other commercial orchards during 2007, 2008, and 2009.

MATERIALS AND METHODS

Field studies were conducted to study the flight bionomics of adult CM in four commercial and four abandoned apple orchards, each with an infestation history of CM during 2007–2008. All orchards were located in Adams County, PA, USA.

Description of Abandoned and Commercial Apple Orchard Ecosystems

All test orchards were in Adams County, PA and were planted with mixed (more than two) cultivars. In abandoned orchards, the tree height was ≈ 7–8 m, and the trees were ≈45+ years old. In commercial orchards, the tree height was ≈ 2.5–3.5 m, and the trees were ≈20+ years old. All abandoned orchards had been neglected for at least 7–15+ years, while the commercial orchards were regularly managed with conventional insecticide programs (Anonymous, 2012). Abandoned orchard sites were at the following locations: Site 1- (39°.49. 1830° N, 77°.22. 3138° W), Site 2- (39°.58. 5260° N, 77°.14. 6138° W), Site 3- (39°.59. 1289° N, 77°.14. 4126° W), and Site 4- (39°.59.7791°N, 77°.14.9361° W), and the commercial sites were at the following locations: Site 1- (39°.50. 2999° N, 77°.11. 4278° W), Site 2- (39°.58. 4723° N, 77°.14. 2756° W), Site 3- (39°.59. 1694° N, 77°.14. 9140° W), and Site 4- (40°. 0. 3478° N, 77°.14. 7020° W). The distance between abandoned and commercial orchards at each site was 1.5–2.5 km, and the distance between the orchards located at Site 1 and the orchards located at Site 4 was ∼40 km.

Monitoring of Adult Codling Moth Populations

Two types of commercially available CM sex pheromone monitoring lures from Trécé Inc. (Adair, OK, USA, 74330), viz. CM Long-Life™ L2™ (hereafter referred to as CM L2) (3.5 mg of (*E, E*)-8,10-dodecadien-1-ol in gray halobutyl septum) and Pherocon® CM-DA Combo™ (mixture of 3.0 mg of (*E, E*)-8, 10-dodecadien-1-ol and 3.0 mg of ethyl (2*E*, 4*Z*)-2, 4 decadienoate in gray halobutyl septum) were used in the study orchards to determine the flight bionomics of CM throughout the growing

season each year. The CM DA Combo lure (hereafter referred as CM DAC) acts as an attractant to both sexes of CM, while the CM L2 lure attracts only male moths (Light et al., 2001; Knight and Light, 2005; Joshi et al., 2011a; Bohnenblust et al., 2011b).

Placements of Traps on Trees and Traps Maintenance

In both types of orchard ecosystems, both lure types were placed in Large Plastic Delta traps (Trécé Pherocon VI®, Adair, OK) attached to bamboo poles (≈2–2.5 m in length) placed in the upper 2/3rd of the tree canopy. Four traps (2 traps/ lure type) were placed in all commercial and abandoned orchards, except the orchards at Site 2, where six traps (3 traps/lure type) were placed. Each trap was placed at ≈90–100 m apart in a row and ≈20–25 m from the border. The trap-tree rows were spaced at least 70 m apart from each other. The traps were placed in all orchards on 23 April and 20 April in 2007 and 2008, respectively, and trap monitoring started approximately 1 week after trap placement. The traps were checked twice a week for moth capture. The effect of trap location and lure type on moth capture was minimized by rotating the position of each trap (in clock-wise manner) to the next trap location within each trap-tree row at every observation period. All captured moths were sexed. The sticky liners of all traps were changed at least every 2 weeks or as needed. Lures were changed after 8 weeks in all orchards, and in both years, all the traps were checked until the end of the season (i.e., mid-October).

Statistical Analysis and Model Development

The percent cumulative moth capture of CM for each orchard was determined and plotted over the accumulated degree-days from biofix (i.e., first sustained moth capture) for each study site to construct models for predicting CM flight. In both years, the biofix was established according to emergence of male moths in all commercial and abandoned orchard ecosystems.

The degree-days for the entire study period were calculated during the moth trapping period for each study site from the daily maximum and minimum temperatures (maximum temperature + minimum temperature/2). High resolution temperature data (<1 km) were obtained from ZedX, Inc. (Bellefonte, PA, 16823). For degree-day calculations, threshold temperatures of 10°C (50°F) and 31°C (88°F) were used as lower and upper thresholds, respectively (Glenn, 1922a,b).

The mean cumulative percent moth capture of CM (after the biofix date) for each generation was used in constructing the logistic flight models for the respective orchards. (Neter and Wasserman, 1974; Knight, 2007):

$$E(Y) = e^{(\beta_0 + \beta_1 * X)}/(1 + e^{(\beta_0 + \beta_1 * X)}) \quad (1)$$

where Y is the proportion of the event completed (mean cumulative percent moth capture), X is the cumulative degree-days from biofix date, β_0 = Intercept and β_1 = Slope. The β_0 and β_1 parameters of the logistic equation were estimated with linear regression by first transforming the proportions (p) into logits (p') as follows (Neter and Wasserman, 1974; Knight, 2007):

$$p' = Log_e[p/(1-p)] \quad (2)$$

Based on the duration of the first and second generation CM flight curves, the following two types of modeling approaches were tested, and compared with the currently used PETE model for predicting CM flight phenology:

I. In the constrained model, the degree-day cutoff (i.e., completion) for the first or second generation flights of CM from the date of biofix (in terms of accumulated DD) was assumed to be identical to that utilized by the current model (i.e., PETE). In both years, the degree-day cutoffs for the constrained models for both the first and second generation flights were assumed as ∼455–465 DD and ∼1055–1060 DD, respectively. This model is termed constrained PCM.
II. In the unconstrained model, the degree-day cutoff for the first or second generation flights of CM from biofix (in terms of accumulated DD) was based on actual moth captures, i.e., end of first or second generation flight as determined from the mean seasonal moth capture graphs (or in other words, complete or near complete shut-down of moth capture in the traps or the lowest trap capture date near the estimated completion date of both generations). In both years, the degree-day cutoffs for this model for both the first and second generation flights were determined to be ∼530–550 DD and ∼1175–1195 DD, respectively. This model is termed unconstrained PCM.

Model Comparisons and Evaluation

The logistic model (for the current model) for CM flight/adult emergence was derived from the tabular values for the PETE model (Brunner and Hoyt, 1982). An analysis of covariance (ANCOVA) was used to determine differences between the slopes and intercepts of the logistic regression equations for both flight models (i.e., constrained and unconstrained PCM) in both commercial and abandoned orchards. The PCM models were compared with the original logistic PETE model for CM flight patterns (in terms of adult emergence) in both types of orchard ecosystems.

Based on the mean seasonal moth capture analyses for both years, the unconstrained PCM model was found to be more representative of CM flight patterns in abandoned and commercial apple orchards in Pennsylvania. Therefore, the PCM unconstrained model was further evaluated for its performance in three different commercial orchards for 3 years (2007, 2008, and 2009). The commercial orchards where the model performance was evaluated were located in Adams County, PA, USA, and received only conventional insecticide applications. The daily minimum and maximum temperatures for these orchards were obtained from the Fruit Research and Extension Center in Biglerville, PA. The R software (R Development Core Team, 2005; ISBN 3-900051-07-0) was used to perform all data analyses including data transformation and logistic modeling, and graphs were

generated in SigmaPlot® 11 (Systat Software, Inc., Chicago, IL USA).

RESULTS

General Seasonal Flight Patterns of CM in Commercial and Abandoned Orchards

Mean seasonal adult moth captures in abandoned and commercial orchards were consistently high in both years, and populations of CM were generally higher in abandoned orchards than in commercial orchards (**Figures 1**, **2**). In 2007, the mean seasonal moth capture in CM L2 baited traps (**Figure 1A**) and CM DAC baited traps (**Figure 1B**) showed a distinct bimodal emergence pattern during the first generation. However, in 2008 (**Figures 2A,B**), the bimodal emergence peaks of first generation were not as distinct as in 2007. During both years at the end of first and second generation flights, the lowest mean seasonal moth capture (degree day cutoff) occurred around ∼550 DD and ∼1230 DD, respectively (**Figures 1**, **2**). In both years, moth capture in CM L2 and CM DAC baited traps showed very similar seasonal patterns for both abandoned and commercial orchard ecosystems (**Figures 1**, **2**). Also, a partial third generation was recorded in both years (**Figures 1**, **2**). In both orchard types, moth flight in both years declined rapidly after ∼1325 DD with the last moths captured at ∼1525 DD (**Figures 1**, **2**).

Codling Moth Male Flight: PCM Model Development in Abandoned and Commercial Orchards

When moth capture data using the CM L2 lures were combined over both years, the flight curves generated by both constrained (**Figures 3A,E**) and unconstrained (**Figures 3C,G**) PCM models were significantly different from the predicted emergence curves generated by the current PETE model for both generations of CM and for both orchard ecosystems ($P = 0.000$; **Tables 1**, **2**). In abandoned and commercial orchards, the PCM constrained (**Figures 3A,E**) and unconstrained (**Figures 3C,G**) models showed differences in the prediction of CM flight curves for both first and second generations at intercept ($P = 0.000$; **Tables 1**, **2**), but not at slope ($P > 0.05$; **Tables 1**, **2**).

In abandoned as well as commercial orchards, the PCM models (both constrained and unconstrained) developed from the data (both years combined data) on moth capture using CM DAC lure baited traps showed significant differences in CM flight curves at slope and intercept for both the first (**Figures 3B,F**) and second (**Figures 3D,H**) generations from the PETE model ($P = 0.000$; **Tables 1**, **2**). The PCM constrained model showed that the flight curves in abandoned orchards vs. commercial orchards were different at intercept and slope ($P < 0.05$) for the first generation and only at intercept for the second generation ($P < 0.05$; **Tables 1**, **2**). In a similar comparison of CM flight curves in commercial and abandoned orchards, the PCM unconstrained

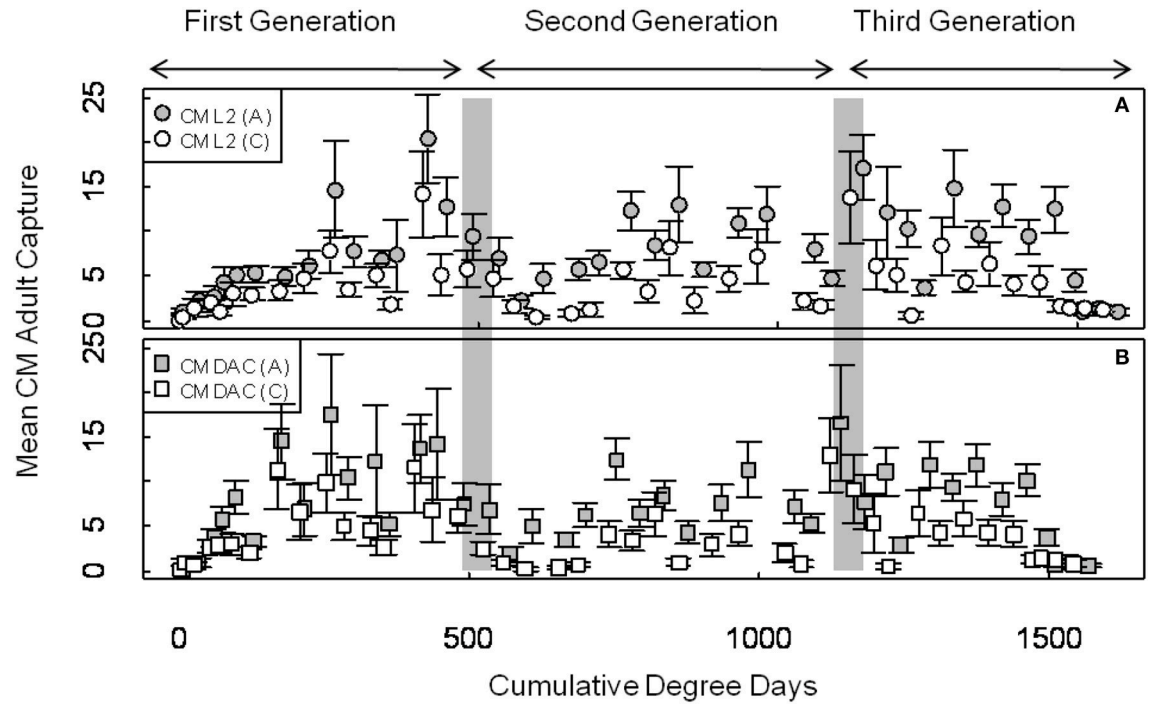

FIGURE 1 | Seasonal flight patterns in terms of mean seasonal moth capture (±SEM) per trap (first and second generation) using CM L2 (A) and CM DAC (B) lures in abandoned (A) and commercial (C) apple orchards in Pennsylvania in 2007. A light gray band on the graph indicates ranges of PETE-CM model/constrained model degree days cutoff for the first and second generations of CM. Degree days (on degree Celsius scale) are cumulative degree days started from the respective date of biofix in both types of orchards.

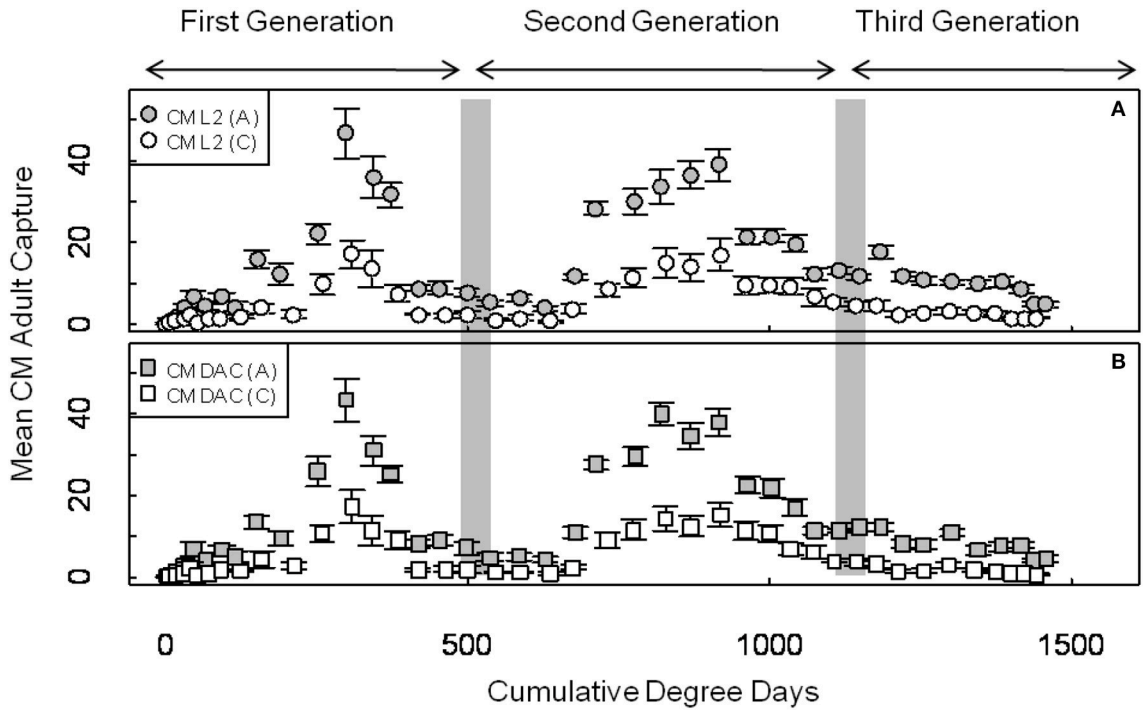

FIGURE 2 | Seasonal flight patterns in terms of mean seasonal moth capture (±SEM) per trap (first and second generation) using CM L2 (A) and CM DAC (B) lures in abandoned (A) and commercial (C) apple orchards in Pennsylvania in 2008. A light gray band on the graph indicates range of PETE-CM model/constrained model degree days cutoff for the first and second generations of CM. Degree days (on degree Celsius scale) are cumulative degree days started from the respective date of biofix in both types of orchards.

model showed significant differences at both intercept and slope of CM flight for both generations (**Tables 1**, **2**).

Comparison of Models Developed from Moth Capture in CM L2 and CM DAC Lures in Abandoned and Commercial Orchards

In abandoned orchards, the constrained PCM flight model curves (developed from moth capture using CM L2 and CM DAC lure baited traps) showed differences in intercept for the first and second generation, but not slope (**Figures 3A,B,E,F**; **Tables 1**, **2**). The unconstrained model approach also showed similar results (**Tables 1**, **2**; **Figures 3C,D,G,H**). In commercial orchards, both constrained and unconstrained PCM models showed differences for both slope and intercept of the first generation and only intercept of the second generation ($P < 0.05$; **Tables 1**, **2**; **Figure 3**).

Female Moth Capture and Flight Model

Mean seasonal female moth captures using CM DAC baited traps in abandoned and commercial orchards were consistently low in both years (**Figures 4A,B**). A female flight prediction model was developed from the percent cumulative female moth capture in CM DAC lure baited traps (**Figures 5A,B**). When the female flight curves of abandoned orchards were compared with the female flight curves of commercial orchards, the model showed significant differences in intercept for the flight curves of both generations (**Table 3**). However, these curves were not different in slope (**Table 3**).

PCM (Male) Flight Model Evaluation (Predicted vs. Observed) and Its Comparative Validation in Commercial Orchards

The ANCOVA analysis showed that the flight predictions from the PCM unconstrained model representing the extended moth emergence pattern was significantly different from the PETE and constrained models (**Tables 1**, **2**). Further, the mean moth capture analysis (**Figures 1**, **2**) showed that the PCM unconstrained model was more representative of the extended moth emergence patterns than the constrained/PETE models for PA apple orchards. Therefore, the PCM unconstrained model was further evaluated for its prediction performance and validated against 3 years of moth capture/emergence data from three different orchards in Adams County, PA. The PCM unconstrained model had the most accurate predictions of moth emergence in all three orchard sites across all 3 years ($R^2 > 0.95$; **Figure 6**). The observed moth capture/emergence data (cumulative moth capture from traps baited with CM L2 and CM DAC lures) for both generations in all orchards and years fell within the 95% prediction interval range/bands of the PCM unconstrained model predictions (**Figure 6**).

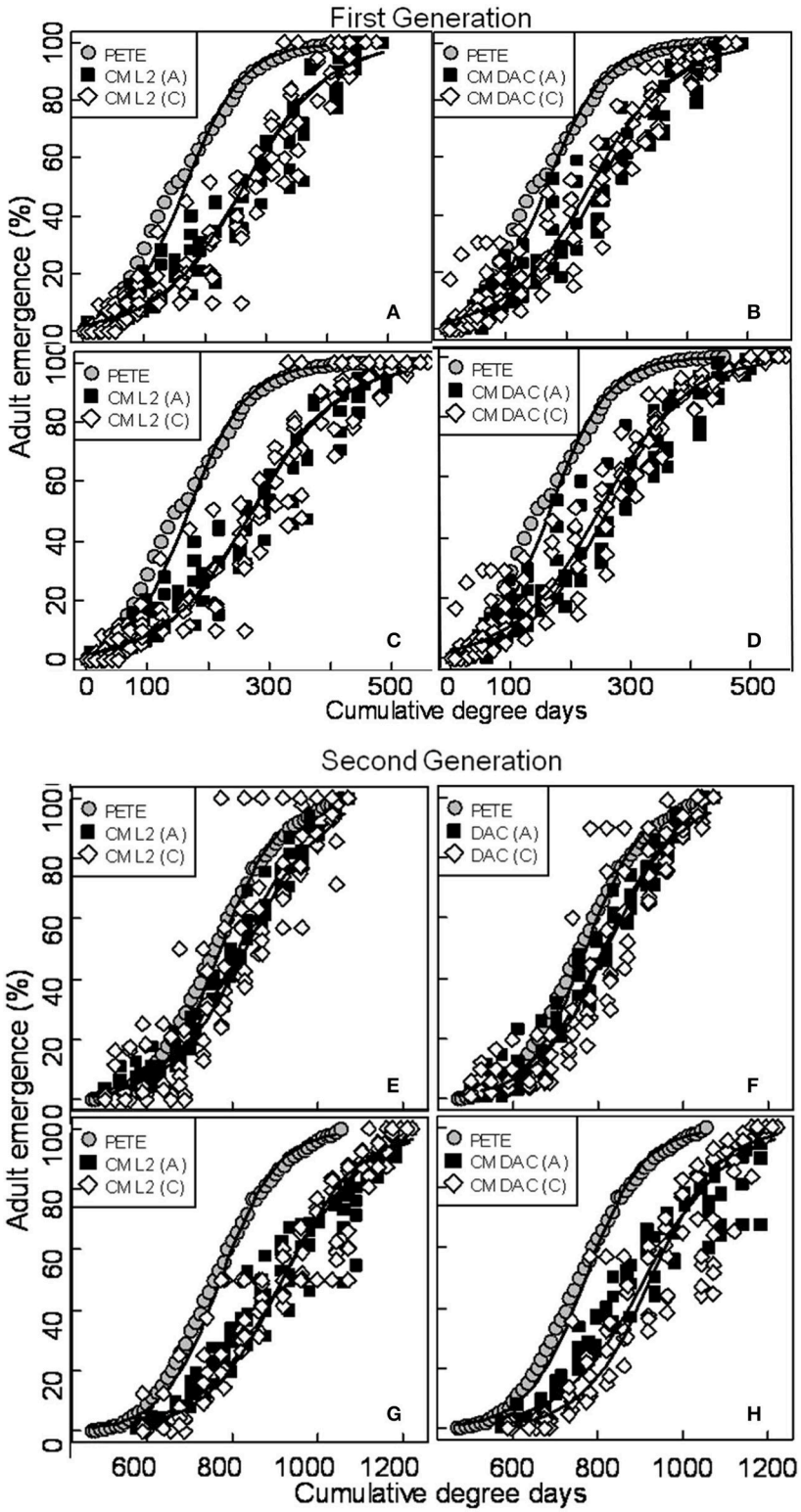

FIGURE 3 | Prediction of seasonal male flight patterns of codling moth in terms of cumulative percent capture of adults [first (A,B,C,D) and second generation (E,F,G,H)] using CM L2 and CM DAC lures in abandoned (A) and commercial (C) apple orchards by constrained, unconstrained and PETE CM logistic models. A comparison of constrained **(A,B,E,F)** and unconstrained **(C,D,G,H)** PCM logistic flight models with PETE-CM model is shown. The models were developed using moth capture data collected during 2007 and 2008. Degree days (on degree Celsius scale) are cumulative degree days started from the respective date of biofix in both types of orchards. In each graph, symbols represent observed data and solid lines represent models.

TABLE 1 | Model parameters of logistic PCM models and PETE model for codling moth (first and second generation) flight in commercial and abandoned orchards.

Lure (orchard type)	Intercept	*SE*	Slope	*SE*	Adjusted R^2
CONSTRAINED MODEL					
First generation					
CM L2 (A)	−3.912	0.113	0.008	0.0003	0.89
CM L2 (C)	−3.755	0.129	0.008	0.0003	0.87
PETE	−3.517	0.133	0.012	0.0003	0.98
CM DAC (A)	−3.987	0.130	0.009	0.0003	0.89
CM DAC (C)	−3.513	0.125	0.008	0.0003	0.88
Second generation					
CM L2 (A)	−9.507	0.204	0.007	0.0002	0.95
CM L2 (C)	−9.439	0.471	0.006	0.0003	0.81
PETE	−11.391	0.199	0.008	0.0001	0.99
CM DAC (A)	−9.756	0.243	0.007	0.0002	0.94
CM DAC (C)	−9.930	0.416	0.007	0.0003	0.84
UNCONSTRAINED MODEL					
First generation					
CM L2 (A)	−3.954	0.106	0.008	0.0002	0.92
CM L2 (C)	−3.799	0.121	0.008	0.0003	0.90
PETE	−3.517	0.133	0.012	0.0003	0.98
CM DAC (A)	−3.978	0.122	0.008	0.0003	0.91
CM DAC (C)	−3.508	0.107	0.008	0.0002	0.92
Second generation					
CM L2 (A)	−9.303	0.206	0.006	0.0001	0.95
CM L2 (C)	−10.290	0.391	0.006	0.0002	0.87
PETE	−11.391	0.199	0.008	0.0001	0.99
CM DAC (A)	−9.392	0.255	0.006	0.0002	0.92
CM DAC (C)	−11.692	0.459	0.007	0.0003	0.87

(A), Abandoned orchard; (C), Commercial orchard.
Cumulative moth capture data was derived from moth capture using CM L2 and CM DAC lures in commercial and abandoned orchards during 2007 and 2008.

For the commercial orchard data in 2007/2008, the newly developed PCM unconstrained model (generated from moth capture data with either CM L2 or CM DAC lure baited traps) predicted a delay in the flight emergence pattern of male adults of ∼30–55 DD at the 10 percent cumulative moth emergence period during the first generation when compared to the predicted moth emergence pattern generated by the CM PETE model (**Figures 3C,D**). When further compared to the PETE model, the PCM unconstrained model also showed a delayed emergence of moths by ∼100–125 DD and ∼120–130 DD at 50 and 90 percent cumulative moth emergence level, respectively (**Figures 3C,D**). During the second generation of CM in commercial orchards, the unconstrained model (irrespective of lure types) showed a delayed emergence by ∼125–140 DD at 10 percent cumulative moth emergence as compared to the predicted moth emergence from the CM PETE model (**Figures 3G,H**). Again, when compared to the PETE model, the PCM unconstrained model also showed a delayed emergence of moths by ∼160–165 DD and ∼150–160 DD at 50 and 90 percent cumulative moth emergence levels, respectively (**Figures 3G,H**).

DISCUSSION

In these studies we found that the currently used CM PETE model was inadequate when predicting CM adult flight in apple orchards of Pennsylvania. We propose a new model, PCM, which more accurately reflects CM flight in Pennsylvania and perhaps elsewhere.

Codling moth flight patterns in both commercial and abandoned orchard types were also different from those predicted by the PETE model. During both years, CM adult populations (in terms of mean seasonal moth capture) were significantly higher in abandoned orchards than nearby commercially managed orchards. The higher populations in abandoned orchards were most likely due to the absence of chemical insecticide programs in these orchards over the previous 5–10 years. Differences in CM abundance between these two orchard ecosystems could also be due to other factors, such as variation in tree canopy size and landscape characteristics. Also, an extended emergence/flight period for male CM was observed for both generations. In both types of orchard ecosystems, adult CM showed a bimodal emergence pattern during their first generation flight and a unimodal emergence pattern during their second generation flight. The delayed or extended emergence/flight pattern during the first generation is likely to cause subsequent delays during the moth flight of the second generation. Due to this extended emergence patterns of male CM, the completion of first and second generation flight (as showed by the mean seasonal moth capture) was delayed by ∼95 and ∼150 DD, respectively, from the predictions generated by the PETE model. The asymmetric emergence patterns (i.e., extended emergence of moths during both generations) of CM could be due to variability associated with diapause induction in CM larvae (Steinberg et al., 1988). A certain proportion of a CM population from each generation enters diapause (Brown et al., 1979), and the full-grown fifth instar CM larvae overwinter in a silken cocoon (Shelford, 1927). The overwintering CM larvae complete their development and life cycle upon arrival of favorable environmental conditions, primarily during the following spring season (Geier, 1963). However, adults from these diapausing larvae do not emerge together (as a cohort) at the onset of the spring season of the next year, but they emerge at different timings (Brown et al., 1979; Steinberg et al., 1988), resulting in differences in the post-diapause spring emergence timings/patterns.

In this study, when the cumulative moth capture data were fit to either a constrained and unconstrained modeling approach, both PCM models conclusively showed a delay in the emergence/flight of moths (irrespective of monitoring lure types and study year) compared to the PETE model. For instance, during the first generation, PCM unconstrained model predicted delays in moth emergence (compared to the PETE model) by ∼30–55 DD, ∼100–125 DD, and ∼120–130 DD at 10, 50, and 90 percent cumulative moth emergence levels, respectively, in commercial orchards. Similarly, delays in moth emergence were also recorded during the second generation in commercial orchards. In both types of orchard ecosystems, the PCM models showed a delayed emergence of CM as

TABLE 2 | Statistical details (ANCOVA *P*-values) of codling moth models comparison in commercial and abandoned orchard ecosystems.

Lure (orchard type)	PCM constrained model				PCM unconstrained model			
	First generation		Second generation		First generation		Second generation	
	Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope
CM L2 (A)								
PETE	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
CM L2 (C)								
PETE	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
CM L2 (A)								
CM L2 (C)	0.000*	0.627	0.000*	0.096	0.000*	0.724	0.000*	0.823
CM DAC (A)								
PETE	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
CM DAC (C)								
PETE	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
CM DAC (A)								
CM DAC (C)	0.000*	0.009*	0.000*	0.313	0.000*	0.013*	0.000*	0.046*
CM DAC (A)								
CM L2 (A)	0.000*	0.352	0.000*	0.959	0.000*	0.354	0.000*	0.417
CM DAC (C)								
CM L2 (C)	0.000*	0.002*	0.000*	0.606	0.000*	0.002*	0.000*	0.285

(A), Abandoned orchard; (C), Commercial orchard.
An asterisk denotes a significant difference at the 0.05 level.
*Model parameters of these CM models are given in the **Table 1**.*

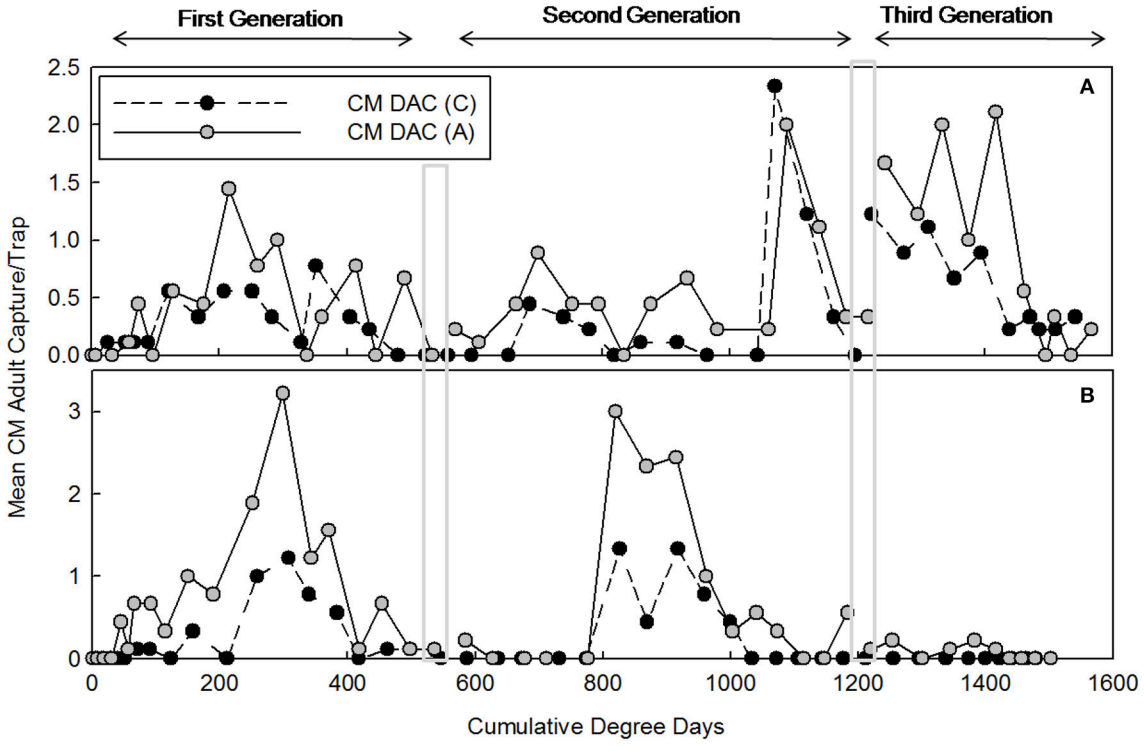

FIGURE 4 | Seasonal female codling moth flight patterns in terms of mean seasonal moth capture (±SEM) per trap (first and second generation) CM DAC lure in abandoned (A) and commercial (C) apple orchards in Pennsylvania in 2007 (A) 2008 (B). A light gray band on the graph indicates range of degree days cutoff for the first and second generations of CM. Degree days (on degree Celsius scale) are cumulative degree days started from the respective date of biofix in both types of orchards.

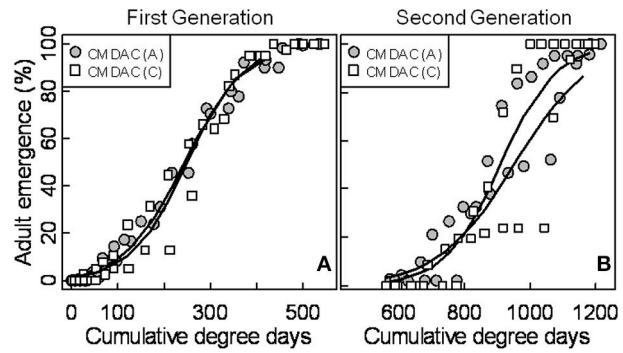

FIGURE 5 | Seasonal flight patterns of female codling moth in terms of cumulative percent capture of adults [first (A) and second generation (B)] using CM DAC lures in abandoned (A) and commercial (C) apple orchards in Pennsylvania. The PCM female flight model was developed using moth capture data collected during 2007 and 2008. Degree days (on degree Celsius scale) are cumulative degree days started from the respective date of biofix in both types of orchards. In each graph, symbols represent observed data and solid lines represent models.

TABLE 3 | Codling moth (first and second generation female) flight in commercial and abandoned orchards using CM DAC lures.

Lure (orchard type)	Intercept	*SE*	Slope	*SE*	ANCOVA *P*-values		
					Adjusted R^2	Slope	Intercept
FIRST GENERATION							
CM DAC (A)	−3.771	0.192	0.016	0.0007	0.95		
CM DAC (C)	−4.129	0.240	0.017	0.0009	0.94	0.000*	0.500
SECOND GENERATION							
CM DAC (A)	−10.730	0.836	0.012	0.0009	0.85		
CM DAC (C)	−8.188	2.160	0.009	0.0002	0.48	0.000*	0.338

(A), Abandoned orchard; (C), Commercial orchard.
An asterisk denotes a significant difference at the 0.05 level.
Model parameters of logistic PCM model developed from 2007 and 2008 datasets and ANCOVA P-values.

compared to model predictions from PETE. Knight (2007) reported that the cumulative flight curves for local populations of first generation CM in Washington State were delayed and different from those reported by the PETE model in Washington. Such differences in the model prediction could be due to many factors including continual use of similar insecticidal chemistries over the last 30–40 years, which can cause resistance in CM populations.

Apple production and pest management programs in the U.S. have changed significantly during the last 30–40 years. The intensive applications of insecticides sprayed onto fruit orchards have caused some CM populations to develop resistance to a number of insecticides (Riedl et al., 1986; Bush et al., 1993; Varela et al., 1993; Knight et al., 1994; Dunley and Welter, 2000; Mota-Sanchez et al., 2008). The PETE multi-species pest complex model (Welch et al., 1978) for CM phenology, which is structured on the distributed delay methods of Manetsch and Park (1974) was developed initially in Michigan in the 1970's for insecticide susceptible (normal) populations in abandoned orchards. Pesticide application timings based on the PETE model have been successful in managing susceptible populations of CM (Riedl et al., 1976; Welch et al., 1978), as the populations would behave in a certain manner that could be predicted through the proxy of day-degrees accumulation. However, the PETE model for pesticide susceptible populations may not accurately predict the phenology of pesticide resistant CM populations, and therefore such models may need to be adjusted accordingly over the time.

In another study, we found conspicuous patterns of higher percent mortality (i.e., more insecticide susceptibility) for male CM collected from abandoned than commercial orchards for both first and second-generation flights in Pennsylvania (Joshi et al. unpublished data), indicating that CM populations in some Pennsylvania commercial apple orchards have developed some level of resistance toward azinphosmethyl, the primary insecticide of choice since the 1960s (Hull L.A., personal comm.). These insecticide resistant populations may develop at different growth rates, as the development of pesticide resistance may cause abnormalities in different life-stages and the life history of CM populations in commercial orchards. Such biological differences could be due to either an increase or decrease in the developmental growth rate by different life stages. Lue (2005) found a 9.8% increase in the egg development time of an organophosphate resistant population of CM, while Knight (2004) reported a delay in the spring emergence of CM and a positive correlation with azinphosmethyl tolerance. Such changes in the developmental time of insecticide resistant populations may alter adult CM emergence and flight patterns over the course of a season in commercial orchards, and this phenomenon may also explain some of the differences found in CM flight and adult emergence patterns in commercial orchard ecosystems.

In this study, the female PCM model predicted a delay in emergence at the initial 3–5% cumulative moth emergence period. This longer delay in the emergence of female moths vs. male moths could be due to protandry. A number of studies have reported that male CM emerge earlier than female moths (Newcomer and Whitecomb, 1924; Tadic, 1957; Putman, 1963), and this protandrous emergence behavior may be as early as 7–12 days before the start of female moth emergence (MacLellan, 1976).

The unconstrained and constrained PCM flight models described in this study are generated with a process-based deterministic logistic model approach. The major advantage of this approach is that it can very precisely predict CM phenology in an orchard that is closely related (particularly in terms of agronomic/cultural orchard practices, topography, pest management practices, and abundance and propensity of CM populations) to the orchards from which the initial biological parameters (e.g., moth capture, oviposition, and egg hatch) were obtained to specify the model. Therefore, applications of CM models (i.e., PCM constrained, unconstrained, and PETE models) based on this approach could be very effective in orchards of a specific type. However, the major weakness of such process-based models is that they ignore the uncertainty associated with CM flight patterns, which

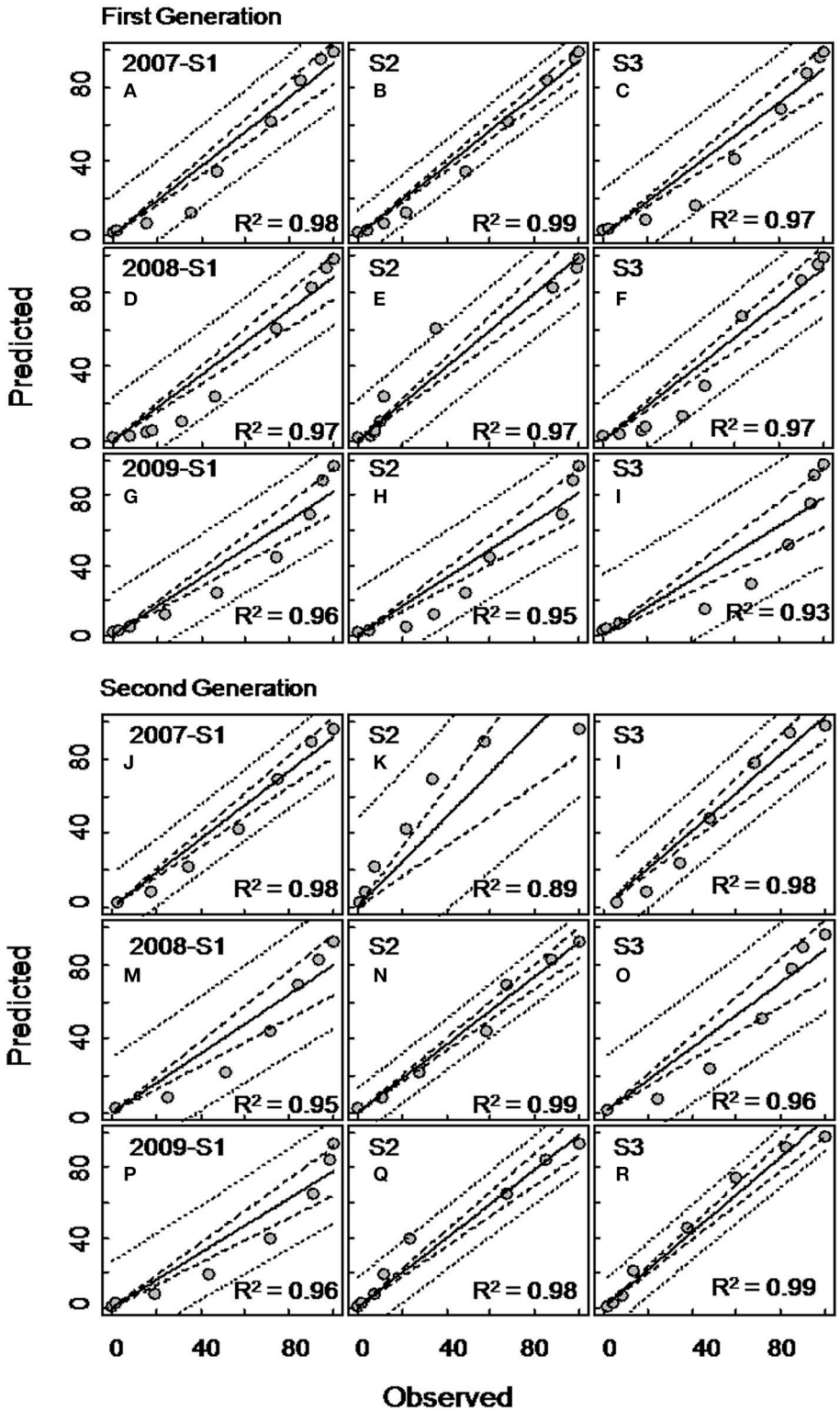

FIGURE 6 | Evaluation of the unconstrained PCM model (developed from moth captures in CM L2 lures baited traps) for codling moth flight prediction for the first (A–I) and second (J–R) generations in three different apple orchards (sites) in Adams County, Pennsylvania during 2007, 2008, and 2009. Each graph of Site 1 (S1), Site 2 (S2), and Site 3 (S3) represents flight patterns in terms of percent cumulative moth capture using commercial lures (combined moth capture in CM L2 and CM DAC baited traps). In each graph, the inner dotted band represents 95% confidence interval and the outer dotted band represents prediction interval.

could encompass factors such as measurement errors, variable climatic conditions, and site/pest management program effects (Joshi et al., 2011b). Application of other modeling approaches (Damos et al., 2011) may be useful in such scenarios.

It is important to note that the PCM constrained and unconstrained models are derived from the cumulative percent moth capture using either the CM L2 or CM DAC lure baited traps in commercial orchards under conventional pesticide spray programs. Therefore, the unconstrained model may not perform well in orchards under sex pheromone mating disruption and other sex pheromone-treated scenarios. Being a deterministic process-based model, it does not consider variability due to the use of sex pheromone mating disruption in apple orchards. Besides the influence of pest management program type, the fit of these models may be significantly influenced by biofix (i.e., 1st sustained moth capture in traps) date. The accuracy of the biofix date at the beginning of moth flight may influence the model prediction performance by either overestimating or underestimating moth flight phenology over the entire season. However, such error in establishing the biofix could be eliminated by using highly attractive lures and monitoring systems, such as those utilized in this study. In both orchard types, two types of commercial lures were used to catch/monitor CM adults, and both types of lures baited traps were checked twice a week, and all the traps were rotated from one location to another to reduce any "hot-spot" based bias (Brunner, 2003). As a result, the error associated with moth capture in the traps was minimized, and both types of lure-baited traps yielded a very similar pattern of moth emergence from the beginning of flight season. However, such error associated with establishing biofix, low moth populations and poor moth capture can be eliminated by starting the accumulation of degree-days from 1 January of each year (Jones et al., 2008). Other aspects such as, variation in a localized strain of CM, level of pesticide resistance, growers' practices, apple cultivars, etc., may also directly or indirectly affect population abundance of CM in apple orchards, and over the time, such factors may significantly alter the completion time of an individual generation of CM. Consequently, all such aspects associated with apple production system may restrict the relative fit and performance of these models.

Studying comparative flight patterns of CM in commercial and abandoned apple orchard ecosystems may reveal some vital information related to the impacts of commercial apple production systems and pest management practices adopted over the past few decades on CM phenology. Applying comparative phenology models (moth capture based on either CM L2 or CM DAC lures) to commercial apple orchards under conventional pesticide programs in Pennsylvania should improve our understanding of CM flights, particularly the puzzling extended emergence and asymmetric flight peaks patterns. Furthermore, if correlated with CM egg hatch timings, the model predictions from the unconstrained CM flight model may also be very helpful in the pesticide spray decision-making process.

AUTHOR CONTRIBUTIONS

NJ, ER, LH and GK designed the experiment. NJ and LH contributed in field work. NJ, LH, KN and ER contributed in data analysis and modeling. NJ prepared the manuscript with contributions from ER, LH, KN and GK. All authors reviewed the manuscript draft.

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Coupling Developmental Physiology, Photoperiod, and Temperature to Model Phenology and Dynamics of an Invasive Heteropteran, *Halyomorpha halys*

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We developed an agent-based stochastic model expressing stage-specific phenology and population dynamics for an insect species across geographic regions. We used the invasive pentatomid, *Halyomorpha halys*, as the model organism because gaps in knowledge exist regarding its developmental physiology, it is expanding its global distribution, and it is of significant economic importance. Model predictions were compared against field observations over 3 years, and the parameter set that enables the largest population growth was applied to eight locations over 10 years, capturing the variation in temperature and photoperiod profiles of significant horticultural crop production that could be affected by *H. halys* in the US. As a species that overwinters as adults, critical photoperiod significantly impacted *H. halys* seasonality and population size through its influence on diapause termination and induction, and this may impact other insects with similar life-histories. Photoperiod and temperature interactions influenced life stage synchrony among years, resulting in an order of magnitude difference, for occurrence of key life stages. At all locations, there was a high degree of overlap among life stages and generation. Although all populations produced F_2 adults and thus could be characterized as bivoltine, the size and relative contribution of each generation to the total, or overwintering, adult population also varied dramatically. In about half of the years in two locations (Geneva, NY and Salem, OR), F_1 adults comprised half or more of the adult population at the end of the year. Yearly degree-day accumulation was a significant covariate influencing variation in population growth, and average maximum adult population size varied by 10-fold among locations. Average final population growth was positive (Asheville, NC, Homestead, FL, Davis, CA) or marginal (Geneva, NY, Bridgeton, NJ, Salem, OR, Riverside, CA), but was negative in one location (Wenatchee WA) due to cooler temperatures coupled with timing of vitellogenesis of F_2 adults. Years of the highest population growth in the mid-Atlantic site coincided with years of highest crop damage reports. We discuss these results with respect to assumptions and critical knowledge gaps, the ability to realistically model phenology of species with strongly overlapping life stage and which diapause as adults.

Keywords: brown marmorated stink bug, phenology, agent-based model, stochastic model, life-history, population dynamics, invasive species

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INTRODUCTION

Phenology modeling of insects typically relies on temperature-dependent functions that describe development as the transition among immature life stages and into the adult life stage. Insect development is often expressed in terms of degree-days (DD), which can be modeled to time management applications, can determine the number of generations across locations, and can be integral in invasive species risk assessments (Damos and Savopoulou-Soultani, 2012). However, DD models ignore life-history functions that are not primarily influenced by temperature, such as the transition between diapausing and non-diapausing life stages. This constrains the utility of single parameter models for insects that overwinter as diapausing adults, where a biofix to initiate DD models is frequently unclear. When used to time management inputs or predict life cycle events in agricultural and forest systems, DD models compensate by establishing a biofix that defines an initial time where development among a subset of life stages, all of which are strongly influenced by temperature, can be initiated. For example, capture of adults in traps is generally used to imply activity including mating or oviposition, which is then used to initiate DD models to estimate the time for development of eggs to more advanced, damaging life stages (Riedl et al., 1976; Wolda, 1988). While this approach has been successful to time management inputs, it limits the scale to the area where this biofix is measured, and requires the ability to define a biofix, often with field measurements.

A variety of insect phenology models have been developed that enable estimation of both diapausing and non-diapausing life stages across wide spatial scales (Gray, 2004; Logan et al., 2007; Powell and Bentz, 2009; Bentz et al., 2014). Phenology models can incorporate physiological variation and additional parameters; one method is through application of agent-based modeling which has been successfully applied to lepidopterans to express the expected distributions of each life stage (Chen et al., 2011; Nealis and Régnière, 2014). Such agent-based methods better approximate realism for a species that has strongly overlapping life stages and heterogeneity in population traits (e.g., egg clutch size, mortality rate, etc.). This approach also enables modeling the distributions of diapausing and non-diapausing adults, which could better define the voltinism potential of insects that overwinter in the adult stage, and effectively captures the interaction of explanatory variables (i.e., photoperiod and temperature) on both development and population dynamics.

We use *Halyomorpha halys* (Stål) (Hemiptera: Pentatomidae) as an model organism to evaluate a stochastic agent-based model where purely temperature-based models might not be appropriate because it overwinters in adult diapause, has strongly overlapping generations, and sampling methods for defining a field biofix are unclear (Nielsen and Hamilton, 2009). As for most temperate insect species, diapause termination, and initiation cues which are influenced by genetics and metabolism as well as extrinsic factors such as temperature and photoperiod (Tauber et al., 1986), have yet to well defined for *H. halys*. Short-day photoperiod as the diapause cue has been identified in many species (reviewed in Koštál, 2011). Utilizing a "standardized" cue such as photoperiod allows insects to keep track of time similar to a circadian clock and evidence is mounting that physiological development post the critical photoperiod is genetically controlled (Bradshaw and Holzapfel, 2010; Koštál, 2011). Species that do so as adults are frequently in a reproductively immature state, which could serve as a physiological adaptation to reduce energy needs during diapause (Numata and Hidaka, 1982; Saunders, 1983; Saunders et al., 1989; Santos et al., 2003). We cautiously assume that diapause termination and induction cues for *H. halys* are the same and are triggered by photoperiod, independent of temperature (Watanabe, 1979; Yanagi and Hagihara, 1980).

As with many other invasives, *H. halys* is a pest that is rapidly expanding its geographic range and pest status in North America and Europe and outbreak densities have caused significant economic loss (Leskey et al., 2012; Rice et al., 2014; Haye et al., 2015; Lee, 2015) and climate niche modeling suggests additional potential expansion (Zhu et al., 2012). *H. halys* overwinters as non-feeding, adults in aggregations on cliff outcroppings, dead upright trees, or human-made structures (Watanabe et al., 1994; Lee et al., 2014) in a non-reproductive (i.e., diapausing) state. The exhibit a gradual emergence from overwintering sites and field estimates on the timing of transition to non-diapausing development and voltinism is difficult due to overlapping generations and a broad host range (Nielsen and Hamilton, 2009; Nielsen unpub.). *H. halys* goes through the egg stage plus five developmental instars before the final molt to adult. Overwintering (diapausing) female *H. halys* are pre-vitellogenic (Nielsen, unpublished), and require an additional period of development prior to sexual maturity. Watanabe (1979) suggests that the critical photoperiod for ovarian development for *H. halys* is between 13.5 and 14.0 h in Toyama, Japan. In temperate latitudes, overwintering *H. halys* adults retain a diapausing physiological state well into the spring before initiation of reproduction (Nielsen, unpublished). Non-diapausing females continue to reproduce throughout their lifespan, which can last up to a few months post diapause emergence (Nielsen et al., 2008; Haye et al., 2014). Other life history components of *H. halys* including pre-ovipositional period, fecundity, nymphal development, and survivorship have been described in relation to temperature for populations in Asia and the US (Yanagi and Hagihara, 1980; Nielsen et al., 2008; Nielsen, unpublished). Although these life table measures are defined in relation to abiotic conditions—and thus do not capture effects due to biotic factors (predation, parasitization, nutrition, etc.)—they provide a basis for modeling the phenology of *H. halys* across a landscape, and for varying landscapes in which this species has recently invaded.

Our objective is to develop a model to estimate voltinism, stage-specific phenology, and population dynamics of an insect with unclear developmental requirements. We do this using *H. halys* as a model organism and predict population dynamics at agriculturally relevant locations across the continental US including locations prior to economically damaging populations. We develop an agent-based model as a synthesis of existing information about *H. halys* life history and demography (e.g., birth and death) as influenced by photoperiod and temperature,

and compare model outputs with field observations. We use these validation efforts, along with varying specific photoperiod sensitivity for diapause induction and termination, to focus attention to areas in need of further study by demonstrating sensitivity of simple population dynamics models to these parameters defining physiology. We then apply the most effective models to eight locations in the US that are relevant to horticultural crops at risk of damage over a 10 year timeframe, which span the latitudes of recent and potential invasion in the US and current climatic conditions.

METHODS

Model Formulation

We adopt an agent-based stochastic modeling framework to explicitly track the life history and population dynamics (through birth and death) of *H. halys* (see Grimm et al., 2006). Each model run is initiated on January 1 with 1000 overwintering female *H. halys*, all categorized as pre-vitellogenic diapausing adults, and continues until the end of the year when the successive generations' adults are in diapause. Each individual *H. halys* is an autonomous agent in this model, and they develop, reproduce, and die according to the environmental factors (temperature and photoperiod). The initial adults are considered the parent generation (P). The model comprises five major modules: (i) diapause termination of generation P, (ii) fecundity of P and successive generations of adults (F_1, F_2, ... F_x), (iii) development from egg to adult, (iv) diapause induction of successive (F_x) adults, and (v) survivorship in all generations (**Figure 1**). Among these five modules, diapause termination and induction are photoperiod-mediated (Yanagi and Hagihara, 1980), while development and survivorship are primarily dependent on temperature (Nielsen et al., 2008). Reproduction is assumed to be mostly environmentally independent, although the newly eclosed adult females need to accumulate about 68 degree-days using a lowest development threshold temperature of 12.7°C (denoted as $DD_{12.7}$) prior to oviposition (Yanagi and Hagihara, 1980). The development module is divided into six stages with varying responses to temperature: from egg to 1st instar nymph (egg incubation), between two consecutive nymphal instars (nymphal development, four processes in total), and between the last (5th instar) nymph and adult (eclosion). We use the temperature-dependent development rates reported by Nielsen et al. (2008), and linear relationships between temperature and development rate for each process computed from these published data (**Table 1**). In each day, each individual *H. halys* has a specific development rate based on temperature and survivorship (Nielsen et al., 2008), and this rate is further applied in a Bernoulli trial to simulate whether the individual will develop to the next stage, and this step imparts stochasticity. For example, two individuals that have exactly the same development rate may result in a different development result (i.e., whether or not transit to next life stage) because of the outcome of the Bernoulli trial. We also use the minimum and maximum developmental thresholds estimated from Nielsen et al. (2008), as 14.17°C and 35.76°C, respectively. Beyond this temperature range, development is halted. In addition, the mean degree-day

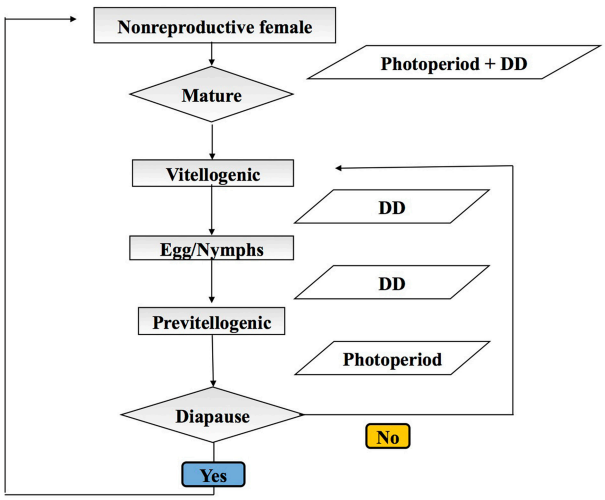

FIGURE 1 | Diagram of the *Halyomorpha halys* individual-based model steps during development. Squares indicate developmental stages and parallelogram indicate inputs. Diapause is represented by a diamond and represents a key physiological decision point.

TABLE 1 | Stage-specific development rate as function of daily mean temperature.

Process	Life stage	Development rate (y = f(T))	R^2
Egg incubation	Egg–1st instar	$y = -0.9843T + 33.438$	0.88
Development	1st instar–2nd instar	$y = -0.3728T + 14.68$	0.84
	2nd instar–3rd instar	$y = -0.6119T + 25.249$	0.81
	3rd instar–4th instar	$y = -0.3986T + 17.602$	0.77
	4th instar–5th instar	$y = -0.4408T + 19.036$	0.82
Emergence	5th instar–Adult	$y = -0.5332T + 24.147$	0.90

$P < 0.01$ for all life stages.
T, daily mean temperature (°C); y, percent development per day for transition through that life stage; e.g., if $y = 0.2$ then the individual has 20% probability of transition to the next life stage.

requirements for egg incubation and complete life history are 53.30 $DD_{13.94}$ and 537.63 $DD_{14.14}$, respectively (Nielsen et al., 2008).

The survivorship module consists of three submodules: for immatures (egg and nymphal stages), for overwintering adults (P), and for all the successive generation adults (F_x). The temperature-dependent immature survivorship rates are reported in Table 2 from Nielsen et al. (2008) and we assume a 1:1 sex ratio. Overwintering adult (P generation) mortality rates post-diapause termination was extracted from Haye et al. (2014). This step also involves stochasticity similar to the development module because of the stochasticity of Bernoulli trial. In addition, eggs and nymphs will die once the daily minimum temperature falls below 0°C after the autumnal equinox. We also make the conservative estimate that reproductive state is not plastic, and thus adults who are reproductively mature ("vitellogenic") prior to the diapause initiation cue of critical photoperiod will not enter a diapausing state, and with all other vitellogenic adults will die at 0°C. In the fecundity module, a total of 68 $DD_{12.7}$

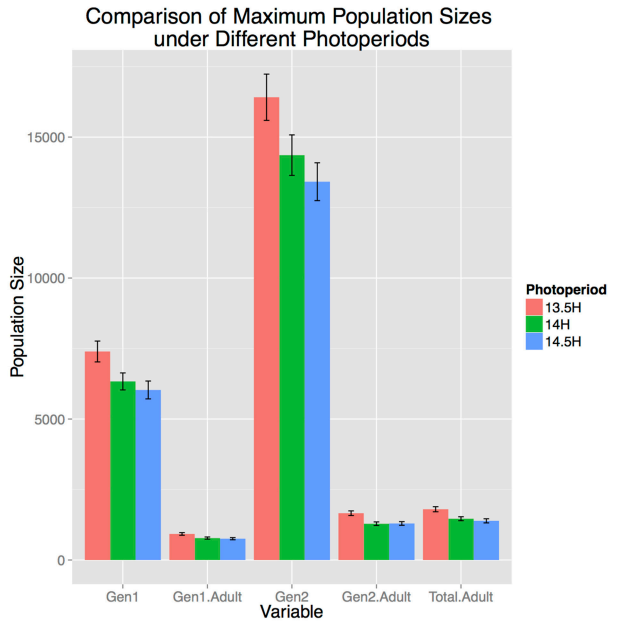

FIGURE 2 | Influence of three photoperiod cues on total population size in Allentown, PA, 2007. Photoperiod units are hours of daylight, and are assumed to influence both diapause termination in the Spring, and diapause initiation after the summer solstice.

is required for the pre-oviposition period (Yanagi and Hagihara, 1980). Clutch size (number of eggs per oviposition, mean ± sem) is 26.08 ± 0.31, the interval between clutches is 4.32 ± 0.41 days, the number of oviposition events is 9.33 ± 0.19, and the number of hatched eggs per clutch are 21.30 ± 0.48, as reported in Nielsen et al. (2008). Each individual vitellogenic female is simulated with a specific oviposition event number, clutch size, and number of hatched eggs (all rounded to the nearest integer). The sex ratio is 1:1.

The model ran for 100 simulations (replication) per site per year (from Jan. 1st to Dec. 31st) to account for stochasticity associated within all the module/submodules. At the beginning of each simulation (Jan. 1st), 1000 overwintering pre-vitellogenic adults were introduced. The model ran at a discrete daily step to check the potential mortality, fecundity, and developmental transition for each individual. The model also kept track of each individual's life stage or phenological category (e.g., whether an individual was in P or F_x generation); and in each specific nymphal and adult reproductive state, and added new births and removed dead individuals to the population. At the end of each simulation, metrics such as daily population size, mean eclosion date for each generation, and degree-day accumulation were collected for further analysis. The model started fresh for a new year (i.e., with 1000 overwintering adults) instead of carrying over from the previous year. All scripts (including all the modules described previously) were written and verified in *R* (v. 3.1.0).

Influence of Photoperiod

The critical photoperiod for diapause termination for *H. halys* is reported to range from 13.5 to 14.75 h (Watanabe, 1979; Yanagi and Hagihara, 1980). We bracketed a critical photoperiod range from 13.5 to 14.5 h, with a 0.5 h step, and compared *H. halys* population dynamics in these three conditions. For simplification, we assume that this critical photoperiod is universal for all individuals in the population (e.g., no individual variation of critical photoperiod cue), and the critical photoperiod for diapause termination prior to the summer solstice is the same as critical photoperiod for diapause induction following the summer solstice. Furthermore, the photoperiod experienced by the F_x adult on the first day of eclosion determines the diapause category for that female. In contrast to the F_x adults, who remain in diapause once they are placed in that category, P adults transition from diapausing to vitellogenic once the critical photoperiod prior to the summer solstice is reached. We chose Allentown, PA (40.40°N, 75.48°W) in 2007 to evaluate the influence of photoperiod by comparing the total and adult population size for the F_1 and F_2 generations under the three photoperiods (13.5, 14.0, and 14.5 h) from 100 simulations using analysis of variance (ANOVA). For model validation and prediction of voltinism across geographic locations we selected the critical photoperiod that resulted in the highest population size as determined by a one-way ANOVA.

Model Validation

Model predictions for Allentown, PA (40.40°N, 75.48°W) were validated using seasonality and density estimates collected with beat sheets from ornamental plants in Allentown, PA, for the years 2005–2007 (see Nielsen and Hamilton, 2009). Life stage-specific numbers of *H. halys* collected twice-weekly were summed across host plants. For validation the population sizes for both observed and predicted populations were scaled relative to the maximum observed population size observed each year and plotted accordingly for comparison. Coefficients of determination (R^2) were computed between field observations and model predictions for three life stages (young nymph, old nymph, and adult) in 3 years (2005–2007).

Phenology and Dynamics at Eight Locations

Subsequent model runs were conducted for the following eight locations representing current and potential areas of *H. halys* invasion across the continental US: Wenatchee, WA (47.42°N, 102.33°W), Salem, OR (44.93°N, 123.03°W), Davis, CA (38.55°N, 121.74°W), Riverside, CA (33.95°N, 117.40°W), Geneva, NY (42.88°N, 76.99°W), Bridgeton, NJ (39.43°N, 75.23°W), Asheville, NC (35.58°N, 85.56°W), and Homestead, FL (25.47°N, 80.47°W). These locations were chosen because of variation in temperature and photoperiod profiles and because they represent locations of significant horticultural crop production that could be affected by *H. halys* in the US. The daily maximum and minimum temperature data were acquired from National Oceanic and Atmospheric Administration (NOAA), and the photoperiod profiles were computed based latitude and day of year following Forsythe et al. (1995). These model runs predicting seasonality and voltinism for these eight locations were conducted for a period of 10 years, from 2005 to 2014, which encompasses current climate conditions and the time period in

which *H. halys* was present in most locations (Leskey et al., 2012; www.stopbmsb.org). This time frame also includes 2010, the year in which *H. halys* populations were very large in the mid-Atlantic region, and years in which temperature extremes occurred. At the end of model simulations for a given location and year, metrics such as mean number of generations, mean and variability of emergence time of various life stages for each generation, and population size (of total individuals and adults), were collected.

The influence of model predictions on population size was analyzed for maximum adult population, final population size (adults entering overwintering diapause) and total DD accumulation through the entire year. We tested for differences in population sizes among locations with analysis of covariance (ANCOVA) in *R* (ver. 3.1.0), assuming results in different years were independent observations for the same location and using year as a covariate, followed by a Tukey's HSD test to explicitly compare sites.

RESULTS

Influence of Photoperiod

Photoperiod strongly influenced population dynamics and abundance. Population development in Allentown, PA occurred between April 20–August 23, May 1–August 10, and May 17–July 26, for the critical photoperiods of 13.5, 14, and 14.5 h, respectively. The 1 h difference in critical photoperiod resulted in almost a 2 month shorter time frame during which reproduction and development could occur. Consequently, shorter photoperiod cues resulted in significantly larger total population size and adult population size in both generations and total population entering diapause ($p < 0.01$, **Figure 2**). The most substantial difference was observed for the F_2 adult population where a 13.5 h photoperiod resulted in 22 and 20% larger population size than a 14.0 or 14.5 h photoperiod, respectively. Furthermore, more southern locations (e.g., Homestead, FL) did not have a photoperiod $\geq$14.0 h and thus a critical photoperiod beyond that was not appropriate for these locations. Among the ranges reported in the literature, 13.5 h of daylength resulted in the largest populations, and thus was the most conservative estimate for considering population growth potential across the entire range of continental US. Therefore, for this study we used a 13.5 h critical photoperiod in the remaining model runs.

Model Validation

Model validation was performed using occurrence data collected in Allentown, PA from 2005 to 2007 on ornamental trees and shrubs of various origin (**Figures 3–5**, see Nielsen and Hamilton, 2009 for data). Population phenology was divided into three life stages, young nymphs (1st–3rd instar), old nymphs (4th and 5th instar), and adults. Model outputs in all 3 years indicate that adult P, F_1, and F_2 generations had overlap although some distinction was observed between young and old nymph stages. In 2005, the predicted population phenology for all three life stages aligned closely with the observed phenology, especially in the timing of the largest population peaks (**Figure 3**). In 2006 for all life stages, and 2007 for large nymphs and adults, the

TABLE 2 | Model outputs defining key population parameters for the years 2005–2014.

Location	Coordinates	Crop	Non-diapause range	P Oviposition		F_1 Eclosion		F_2 Eclosion		Final adult population	
				Range	Median	Range	Median	Range	Median	F_1	F_2
Geneva NY	42.88°N 76.99°W	Apple	Apr 18–Aug 26	May 30–Jun 17	Jun 7	Jun 21–Jul 18	Jun 30	Jul 19–Aug 9	Jul 28	243–447	81–1847
Bridgeton NJ	39.43°N 75.23°W	Peach/Vegetable	Apr 22–Aug 22	Jun 3–Jun 6	Jun 4	Jun 9–Jul 2	Jun 23	Jul 6–Jul 31	Jul 24	140–278	531–2027
Asheville NC	35.58°N 85.56°W	Tree fruit/Vegetable	Apr 28–Aug 17	May 28–May 29	May 29	Jun 3–Jun 11	Jun 6	Jun 25–Jul 4	Jul 2	89–253	803–2287
Homestead FL	25.47°N 80.47°W	Tomato/Strawberry	May 24–July 22	Jun 5–Jun 6	Jun 6	Jun 17–Jun 18	Jun 18	Jul 8–Jul 10	Jul 9	44–137	1318–2781
Wenatchee WA	47.42°N 102.33°W	Apple/Pear	Apr 14–Aug 31	Jun 7–Jun 23	Jun 10	Jun 11–Jul 4	Jun 30	Jul 18–Aug 7	Jul 22	0–7	13–43
Salem OR	44.93°N 123.03°W	Tree fruit/Wine grape	Apr 17–Aug 28	Jun 8–Jun 9	Jun 9	Jun 17–Jul 2	Jun 26	Jul 24–Jul 31	Jul 27	269–716	33–1035
Davis CA	38.55°N 121.74°W	Tomato	Apr 24–Aug 20	Jun 3–Jun 4	Jun 4	Jun 14–Jun 19	Jun 16	Jul 6–Jul 15	Jul 11	75–255	733–1893
Riverside CA	33.95°N 117.40°W	Citrus	May 1–Aug 13	Jun 10–Jun 11	Jun 11	Jun 20–Jun 24	Jun 23	Jul 8–Jul 16	Jul 13	21–95	349–962

timing of the largest population sizes for all three life stages also aligned closely with the field observations (**Figures 4**, **5**). However, modeled estimates of the timing of populations in the early season were inconsistent. In 2006 for all three life stages, and in 2007 for old nymphs and adults, modeled output predicted higher values earlier in the season. Conversely, in 2007, the model did not capture field estimates of an early season spike in young nymphs. The R^2 values ranged between 0.82 and 0.96 (except young nymphs in 2007 where the R^2 was 0.35) for various life stages in 3 years, showing a robust prediction from the model output comparing to the field observations.

Phenology and Dynamics at Eight Locations

H. halys adults emerged from diapause in a pre-vitellogenic status (**Figures 6**, **7**, **Figure S1**). The timing of diapause termination (noted as the earliest date of entering a non-diapause condition) ranged from April 14 to May 24 (**Table 2**) with the earliest dates being from April 14–18 at latitudes 42.88°–47.92°N. The latest diapause termination date is expected to be May 24 in Homestead, FL. Diapause termination followed a North to South gradient and diapause initiation followed a South to North gradient as would be expected for a short-day diapausing insect species. This invariant response of diapause to photoperiod

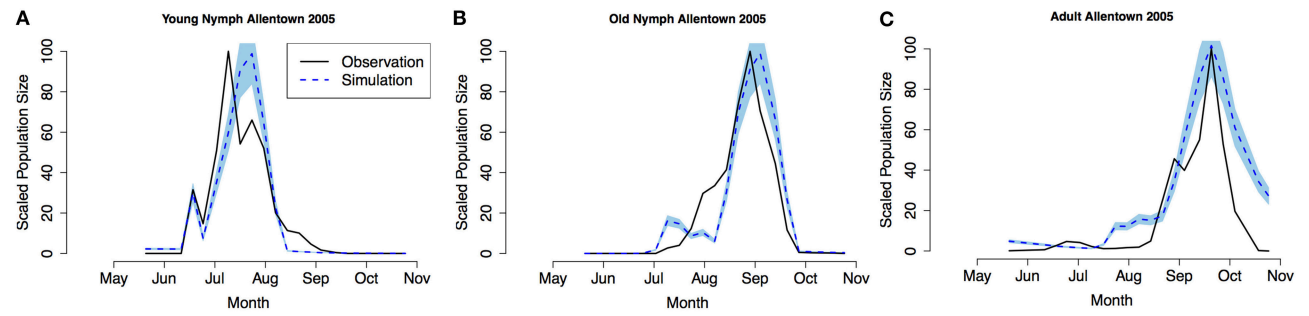

FIGURE 3 | Model validation in Allentown, PA 2005 (A) young nymphs (1st–3rd instar), (B) old nymphs (4th—5th instar), (C) adult. Shaded area represents 95% CI (confidence interval) for predictions from 100 simulations.

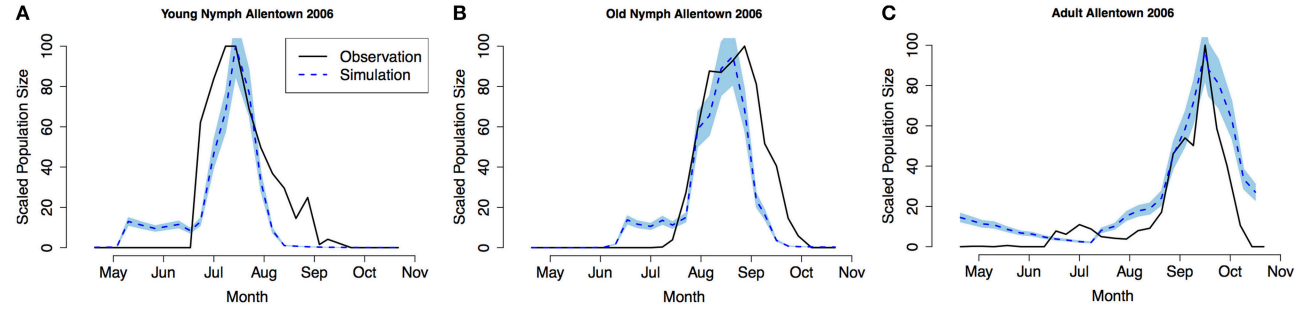

FIGURE 4 | Model validation in Allentown, PA 2006 (A) young nymphs (1st–3rd instar), (B) old nymphs (4th–5th instar), (C) adult. Shaded area represents 95% CI.

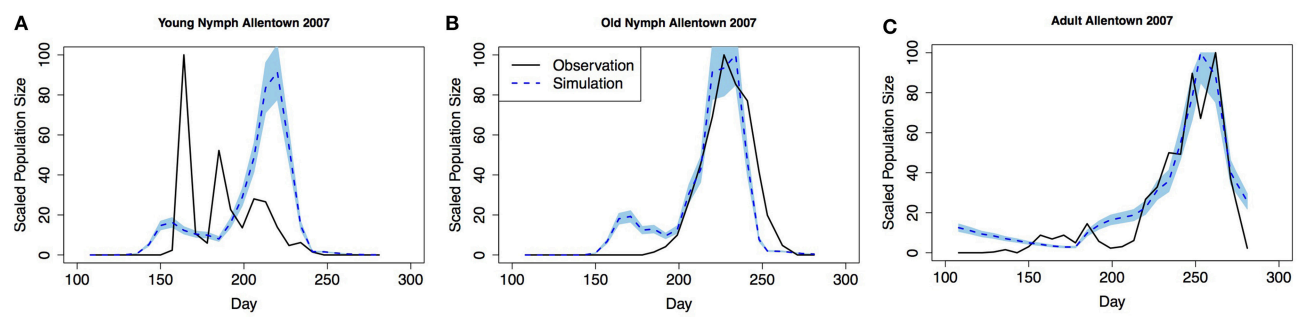

FIGURE 5 | Model validation in Allentown, PA 2007 (A) young nymphs (1st–3rd instar), (B) old nymphs (4th–5th instar), (C) adult. Shaded area represents 95% CI.

strongly influenced population phenology and dynamics, as discussed below, and thus model assumptions tied to diapause represents a key knowledge gap.

Initial oviposition (P oviposition in **Table 2**) occurred rapidly once the conditions were met, and narrowed the variation in population phenology among locations and years. Whereas, the initiation of vitellogenesis occurred over a 40 d span among locations, parental oviposition was initiated over a 26 d time span (between May 28 and June 23 among locations). Thus, median P generation oviposition was estimated to occur within a 2 week time span (May 29–June 11 among all locations and years). However, variation in temperature conditions among locations after critical photoperiod resulted in a bimodal pattern of the range over which oviposition by P generation adults occurred: most locations initiated oviposition during a rapid (1–3 d) time frame, whereas the two northernmost locations initiated oviposition over a 16 d (Wenatchee, WA) or 18 d (Geneva, NY) time span.

Development of the five immature life stages was directly influenced by temperature and resulted in eclosion of the F_1 generation across a narrow temporal distribution in some locations, but much wider range in others. For example, F_1 eclosion occurred between June 14–June 19 (5 days) in Davis, CA, and June 9–July 2 (23 d) in Bridgeton, NJ, despite being initiated at a similar time frame and being at similar latitudes **Figures S6**, **S7**. Although these differences among locations narrowed as the season progresses, they persisted, and result in a 9 d time span for F_2 eclosion in Davis CA, compared to a 25 d time span for F2 eclosion in Bridgeton NJ (**Table 2**, **Figures S2**–**S5**).

At all geographic locations, F_2 adults were predicted to occur and enter into diapause, thus populations at all locations could be characterized as bivoltine. Adults, male or female, that became reproductively mature prior to experiencing the diapause initiation cue were killed upon the first incidence of frost, as were all developing nymphs. However, depending on the timing of eclosion, some F1 adults became reproductive and others were conditioned for diapause. some F_1 adults also eclosed after the critical photoperiod following the summer equinox, and thus there was a mix of both F_1 and F_2 adults entering diapause at the end of the year (**Figures 6**, **7**). The relative contribution of each generation adults to the total, or overwintering, population varied dramatically across the US. For example, in five locations (Asheville, NC, Bridgeton, NJ, Homestead, FL, Davis, CA, Riverside, CA) F_2 adults typically comprised the overwhelming majority of the adults at the end of the year. In contrast, F_1 adults comprised a large fraction of the population at the end of the year in multiple years

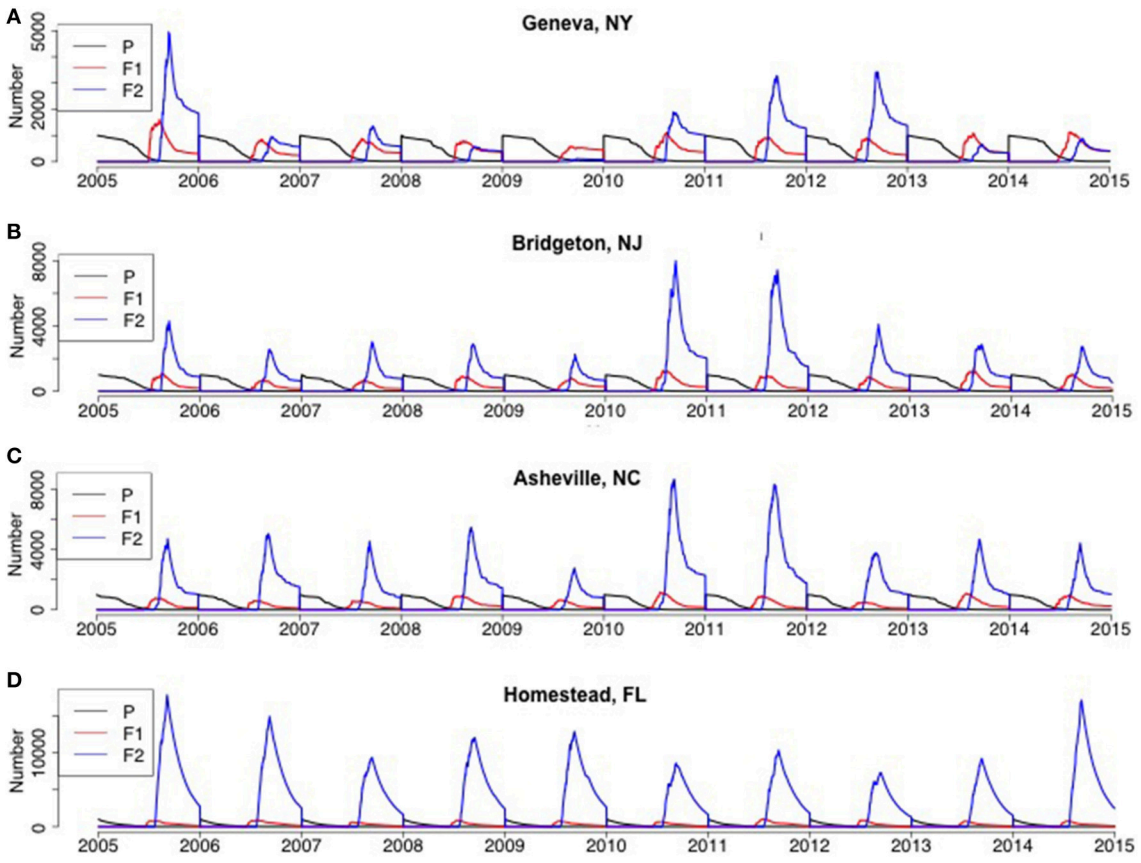

FIGURE 6 | Model predictions of adult population size by generation for the Eastern US (A) Geneva, NY, (B) Bridgeton, NJ, (C) Asheville, NC, (D) Homestead FL from 2005 to 2014. P represented parental overwintered adults, which was initialized as 1000 each year and for each simulation run.

in Geneva, NY and Salem, OR. The F_1 adults comprised an equal or higher fraction than the F_2 adults in 4 of 10 years in Geneva, NY (2008, 2009, 2013, and 2014) and 6 of 10 years in Salem, OR (2005, 2007, 2008, 2010, 2011, and 2012). A more detailed quantitative description of the range in F_1 and F_2 adults at the end of the year for each location is provided in **Table 2**.

There was significant ($p < 0.01$, using ANCOVA) variability of maximum population size and final population size by location (**Figure 8**). Yearly DD accumulation also served as an important covariate and there was a positive correlation ($\rho > 0.8$) between DD accumulation and maximum and final population size, with the only exception of Riverside, CA and Davis, CA, where Davis had higher population size despite lower yearly DD accumulation. This indicated that yearly DD accumulation was an important factor for predicting *H. halys* population sizes. Homestead, FL and Davis, CA had the highest mean maximum adult size in the 10-year period (both over 10,000 individuals) and not significantly different ($p = 0.12$) based on the Tukey's HSD test (**Figure 8A**). On average, positive population growth, (>1000 individuals), was seen in Asheville, NC, Homestead, FL and Davis, CA (**Figure 8B**). Other locations (Geneva, NY, Bridgetown, NJ, Salem, OR, and Riverside, CA) either had marginally positive population growth or marginal population declines. The range of final population sizes at all locations, however, encompassed a doubling or tripling in size, except in Wenatchee, WA. Wenatchee, WA is predicted to be unable to sustain population growth based on 10-year historical data (**Figure 8B**, **Table 2**). Wenatchee, WA also had the lowest accumulation in DD for *H. halys* development (**Figure 8C**, **Figure S5**).

DISCUSSION

We have developed an agent-based stage-specific model for the invasive *H. halys* that incorporates existing physiological information on development, survivorship and reproduction to predict population size, phenology and voltinism across a large geographic scale in the US. It is expected that this model could be applied to other insect species that share similar life history traits, specifically adult diapause and overlapping generations.

The model predicts bivoltinism potential across geographic locations but incorporation of the 13.5 h critical photoperiod has significant impacts on resulting population size across locations. This could influence the establishment and pest potential at locations where population growth was not positive (i.e., Wenatchee, WA). Despite a narrow window for development in the southern locations of Homestead, FL significant population growth is possible. Riverside, CA also has high DD accumulation

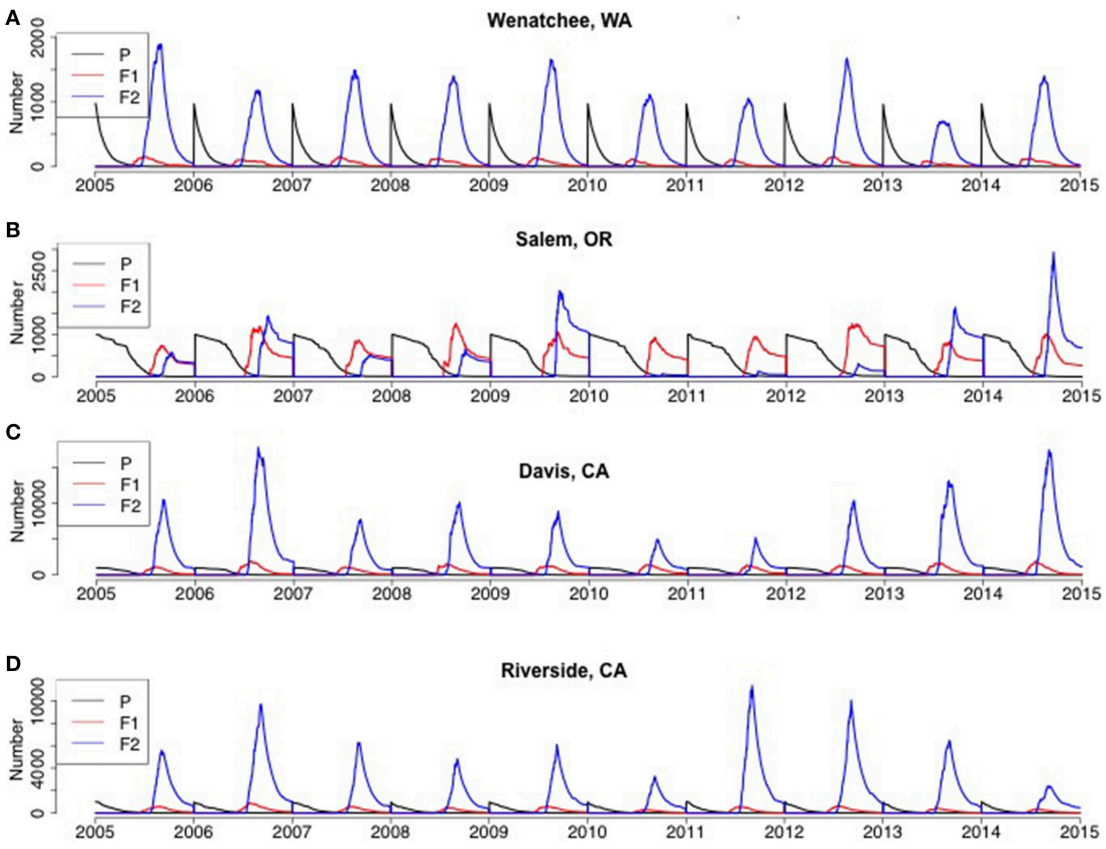

FIGURE 7 | Model predictions of adult population size by generation for the Western US (A) Wenatchee, WA, (B) Salem, OR, (C) Davis, CA, (D) Riverside, CA from 2005 to 2014. *P* represented parental overwintered adults, which was initialized as 1000 each year and for each simulation run.

but was predicted, on average, to not have sustainable population growth. This is likely due to the T_m threshold being reached, which is a limiting factor for temperate insect species. Research by Taylor et al. (2014) suggests that gut symbionts present on *H. halys* eggs also have a maximum temperature and exposure of eggs beyond T_m has impacts on hatch rate and fitness for multiple generations. Currently, this model could be used to redefine risk assessment scenarios and help guide management priorities throughout its invaded range and could be adapted to a web-interface for real-time predictions of phenological events for management purposes.

Population estimates based on visual observations and aggregation pheromone trap collections are highly variable and suggest *H. halys* is typically at low densities early in the spring (Nielsen and Hamilton, 2009; Leskey et al., 2012), which clouds the identification of a biofix and utility of DD models, and influenced our validation efforts. Our phenology model in Allentown, PA differed from observed estimates in the early season (May–June). This is likely due to difficulties finding widely dispersed clumped distributions of *H. halys* as they emerge from overwintering sites at low densities, including significant fractions in trees and human-built shelters (Rice et al., 2014). However, by mid and late season (July–October), the temporal patterns from predicted populations using a 13.5 h photoperiod biofix were well aligned with observed populations in all 3 years. Also, the model projected higher than average populations in Bridgeton, NJ in 2010 and 2011 (**Figure 6**, **Figure S4**), which aligns well with the dramatic increase in crop damage that occurred in the mid-Atlantic region in those years (Leskey et al., 2012).

A 13.5 h photoperiod is within, but at the lower end, of the reported range (Watanabe, 1979; Yanagi and Hagihara, 1980), and is consistent with insects that are in diapause during short-day conditions. Our current modeling efforts utilized a 13.5 h photoperiod for diapause termination and induction because it aligned well in the validation efforts, and provided a wider time scale for reproductive activity, resulting in the most conservative (e.g., highest) prediction for estimating potential

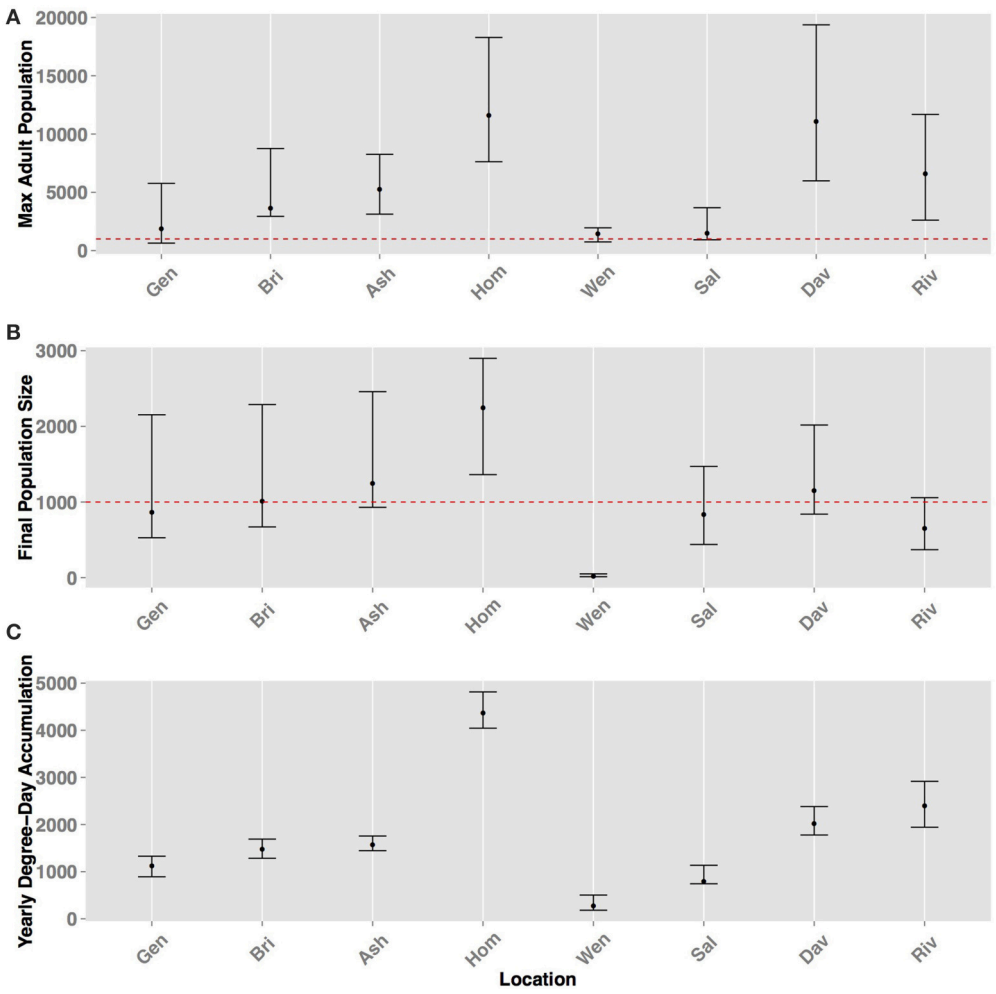

FIGURE 8 | Predicted population sizes (± range from all simulations and years) across all geographic locations for (A) maximum adult population size (B) final population size, and (C) yearly degree-day accumulation. The error bars represent the standard errors from 100 simulations for the metrics.

population growth and voltinism at these temperate-climate locations. However, model predictions are clearly sensitive to assumptions about diapause termination in the spring, diapause induction after the summer equinox, and the maintenance of diapause in adults. Gray et al. (2001) effectively modeled diapause using two co-occurring temperature dependent processes, and it is highly probable that diapause in *H. halys* is influenced by multiple, and interacting, processes as opposed to the single process we utilized.

Model behavior was also strongly influenced by the assumption of the inability of vitellogenic adults to revert to a diapause state (a "lack of diapause plasticity"), and that this absence of diapause in vitellogenic adults resulted in death when temperatures dropped below freezing. In Wenatchee, WA, DD accumulation was the lowest among all sites, resulting in low population densities (**Figure 8**, **Figure S5**, note lower range in y-axis compared to other sites). In addition, the long interval between termination and initiation of diapause, due to the high latitude, was sufficient to enable temperature accumulation for development of F_2 adults, most of which occurred prior to the critical photoperiod that would move them into diapause. Thus, these vitellogenic F_2 adults were subject to the mortality rates applied to all adults, and killed by temperatures below 0°C. The lower densities coupled with the timing of F_2 adults into vitellogenic status resulted in very low and possibly unsustainable populations by the end of the year (**Figures 7**, **8B**). Many species have demonstrated plasticity in their response or rapid evolution to different critical photoperiods (Saunders et al., 1989). Although this has not been demonstrated for *H. halys*, the evolution of short-day response to photoperiod cues for diapause was demonstrated within a few decades of introduction for the invasive mosquito *Aedes albopictus* in the US and is an important adaptation to climatic variation in its invaded range (Urbanski et al., 2012). Pitcher-plant mosquitoes, *Wyeomyia smithii*, *also* demonstrate plasticity in photoperiodic response under climate change scenarios with a more pronounced shift in critical photoperiods in northern latitudes (Bradshaw and Holzapfel, 2001). An additional important assumption of the *H. halys* model is that the diapause cues are consistent across geographic locations, which is in agreement with the small founding population of the Eastern US population but these cues are under significant selection pressure (Xia et al., 2012; Xu et al., 2014). Thus, if *H. halys* evolves a differential response to photoperiod as seen with *A. albopictus*, or haplotypic diversity that results in varying diapause characteristics, the model predictions about population size and growth would change significantly and may expand its range and pest potential.

Agent-based approaches could further refine CLIMEX-based distribution models. Zhu et al. (2012) estimated a disjunct establishment probability of *H. halys* in the US with higher establishment probabilities in the mid-Atlantic, extending into the mid-West and upper South, and the West Coast. This assumes that the existing US populations carry the adaptive potential expressed throughout the geographic range of the insect in Asia. While our phenology model predicts very limited population growth in most years in areas such as Wenatchee, WA and Geneva, NY, it does predict large population growth in more southern locations such as Davis, CA and Homestead, FL, which is in conflict with the climate-matching model. Current populations in Davis, CA anecdotally support our model's prediction and were estimated by the application of additional parameters by Zhu et al. (2012).

Our model has many implications for prioritizing management. Management of *H. halys*, as in other Pentatomids, is difficult due to extensive movement among hosts, landscape elements, and difficulties in field estimates of population densities (McPherson and McPherson, 2000; Nielsen and Hamilton, 2009; Wallner et al., 2014). Field sampled or modeled estimates for a field or orchard are less accurate or stable as individuals from other locations and overlapping life stages in the landscape immigrate or emigrate from that location. Our modeled predictions shows very strong overlap of multiple life stages, and generations, with the co-occurrence of P, F_1, and F_2 generation adults. However, the time span at which life stages overlapped, and degree of synchrony among years for a life stage, varied by location. Southern and warmer locations tended toward greater synchrony among years. For example, F_2 eclosion spanned 2 days in Homestead, FL, compared to 21 days in Geneva, NY. This has implications for timing management directed at specific life stages, and for optimizing sampling efforts. Given the significant population growth observed at most sites, it also emphasizes the importance of managing the P and F_1 generations **Figures S6**, **S7**. Although populations were bivoltine at all locations, simply defining the voltinism potential at a location as a fixed number becomes confounding and can be misleading. In some locations and years (for example Salem, OR) F_2 adults occurred, but at very low numbers, and it was F_1 adults that contributed most strongly to the population at the end of the season. Insect management programs that aim to reduce region-wide populations by targeting overwintering adults, as proposed by Cross (1973) and later accomplished for the boll weevil eradication program, could start much earlier in locations where F_1 adults were a significant fraction of the overwintering population. Field estimates are not able to distinguish among P and F_x adults, thus a modeling framework that effectively expresses distributions of all life stages becomes useful for management.

Importantly, this model does not address all factors that influence insect population phenology or dynamics. We present this model as a synthesis of current understanding of primarily abiotic factors (temperature, photoperiod) influencing *H. halys* phenology and potential population growth. However, our approach aligns well with field observations in Allentown, PA and suggests that our model has sufficient realism to warrant extrapolation to other locations and years. The agent-based approach we utilized could be applied to other insect species with overlapping generations and which diapause in the adult or other life stages. Recently, agent-based modeling has been applied to a few insect models, and phenology models have been applied to consider effects of climate change. Here, we extend the application to addressing invasive species. We present results under current climate conditions, and the model can be readily adapted to extrapolate to future climate scenarios.

AUTHOR CONTRIBUTIONS

All three authors contributed equally to the formulation of the ideas, review of data and the analysis. SF initiated this project. SC conducted the modeling based off of parameters and data derived from AN with input and interpretation by SF. Both AN and SF contributed to evaluation of model outputs and decisions about physiological characteristics. All three authors contributed to drafting and editing the manuscript. This is a truly collaborative effort.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00165

Figure S1 | Simulated population size and phenology of adults transitioning out of reproductive diapause in the Spring and into reproductive diapause in the Fall in Allentown, PA, 2007. Simulation initiation with 1000 overwintered parental adults.

Figure S2 | Predicted total population size by life Stage for the Eastern US (A) Geneva, NY, (B) Bridgeton, NJ, (C) Asheville, NC, (D) Homestead, FL, from 2005 through 2014. Populations were initialized with 1000 adults for each year and simulation run.

Figure S3 | Predicted total population size by life Stage for the Western US (A) Wenatchee, WA, (B) Salem, OR, (C) Davis, CA, (D) Riverside, CA from 2005 through 2014. Populations were initialized with 1000 adults for each year and simulation run.

Figure S4 | Degree-day accumulation for *Halyomorpha halys* development in the Eastern US (A) Geneva, NY, (B) Bridgeton, NJ, (C) Asheville, NC, (D) Homestead, FL, from 2005 through 2014.

Figure S5 | Degree-day accumulation for *Halyomorpha halys* development in the Western US (A) Wenatchee, WA, (B) Salem, OR, (C) Davis, CA, (D) Riverside, CA from 2005 through 2014.

Figure S6 | Predicted total population size by generation for the Eastern US (A) Geneva, NY, (B) Bridgeton, NJ, (C) Asheville, NC, (D) Homestead, FL, from 2005 through 2014.

Figure S7 | Predicted total population size by generation for the Western US (A) Wenatchee, WA, (B) Salem, OR, (C) Davis, CA, (D) Riverside, CA from 2005 through 2014.

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CORRECTION
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Corrigendum: Coupling Developmental Physiology, Photoperiod, and Temperature to Model Phenology and Dynamics of an Invasive Heteropteran, *Halyomorpha halys*

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Keywords: brown marmorated stink bug, phenology, agent-based model, stochastic model, life-history, population dynamics, invasive species

A corrigendum on

Coupling Developmental Physiology, Photoperiod, and Temperature to Model Phenology and Dynamics of an Invasive Heteropteran, *Halyomorpha halys*
by Nielsen, A. L., Chen, S., and Fleischer, S. J. (2016). Front. Physiol. 7:165. doi: 10.3389/fphys.2016.00165

In this manuscript, we present model results from eight locations over 10 years based on temperatures at weather stations and photoperiod. We inadvertently made a poor choice for a weather station to represent Wenatchee WA. To avoid heat island effects, we chose the Grouse Camp weather station to represent Wenatchee. However, although Grouse Camp is only 21 km from Wenatchee, it is in a mountainous area (1,642 m elevation) and poorly represents the climate in the apple growing region of Wenatchee, WA (190 m elevation). Thus, the results do not reflect potential population dynamics of *H. halys* in Wenatchee. In re-evaluation of the model, the data show that populations at Wenatchee, WA, are predicted to behave similarly to those at Salem, OR, with an average albeit marginal positive population growth. Conclusions that were driven heavily by photoperiod, such as the range in days for initiation of oviposition by overwintered adults, were less affected (from 16 down to 13 days). The strong differences were due to markedly higher degree day accumulations at Wenatchee versus Grouse Camp.

The following six files use the same order of tables and figures from the original manuscript and use the Weather Station data for Wenatchee, WA (network ID: GHCND:USC00459074).

- **Table 2**. Model outputs defining key population parameters for the years 2005–2014.
- **Figure 7**. Model predictions of adult population size for Wenatchee, WA. *P* represented parental overwintered adults, which was initialized as 1000 for each year and for each simulation run.
- **Figure 8**. Predicted population sizes (+/− range from all simulations and years) across all geographic locations for **(A)** maximum adult population size, and **(B)** final population size, and **(C)** yearly degree-day accumulation. The error bars represent the standard errors from 100 simulations for the metrics.

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- Figure S3. Predicted total population size by life stage for Wenatchee, WA from 2005 through 2014. Populations were initialized with 1000 adults for each year and simulation run.
- Figure S5. Degree-day accumulation for *Halyomorpha halys* development in Wenatchee, WA from 2005 through 2014.
- Figure S7. Predicted total population size by generation for Wenatchee, WA.

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Weather Station: Wenatchee, WA (Network ID: GHCND:USC00459074).

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TABLE 2 | Model outputs defining key population parameters for the years 2005-2014.

Location	Coordinates	Crop	Non-diapause range	P Oviposition		F_1 Eclosion		F_2 Eclosion		Final adult population	
				Range	Median	Range	Median	Range	Median	F_1	F_2
Geneva NY	42.88°N 76.99°W	Apple	Apr 18–Aug 26	May 30–Jun 17	Jun 7	Jun 21–Jul 18	Jun 30	Jul 19–Aug 9	Jul 28	243–447	81–1,847
Bridgeton NJ	39.43°N 75.23°W	Peach/Vegetable	Apr 22–Aug 22	Jun 3–Jun 6	Jun 4	Jun 9–Jul 2	Jun 23	Jul 6–Jul 31	Jul 24	140–278	531–2,027
Asheville NC	35.58°N 85.56°W	Tree fruit/Vegetable	Apr 28–Aug 17	May 28–May 29	May 29	Jun 3–Jun 11	Jun 6	Jun 25–Jul 4	Jul 2	89–253	803–2,287
Homestead FL	25.47°N 80.47°W	Tomato/Strawberry	May 24–July 22	Jun 5–Jun 6	Jun 6	Jun 17–Jun 18	Jun 18	Jul 8–Jul 10	Jul 9	44–137	1318–2,781
Wenatchee WA	47.42°N 120.33°W	Apple/Pear	Apr 14–Aug 31	Jun 2–Jun 15	Jun 8	Jun 17–Jun 29	Jun 22	Aug 1–Aug 18	Aug 6	106–559	46–1,557
Salem OR	44.93°N 123.03°W	Tree fruit/Wine grape	Apr 17–Aug 28	Jun 8–Jun 9	Jun 9	Jun 17–Jul 2	Jun 26	Jul 24–Jul 31	Jul 27	269–716	33–1,035
Davis CA	38.55°N 121.74°W	Tomato	Apr 24–Aug 20	Jun 3–Jun 4	Jun 4	Jun 14–Jun 19	Jun 16	Jul 6–Jul 15	Jul 11	75–255	733–1,893
Riverside CA	33.95°N 117.40°W	Citrus	May 1–Aug 13	Jun 10–Jun 11	Jun 11	Jun 20–Jun 24	Jun 23	Jul 8–Jul 16	Jul 13	21–95	349–962

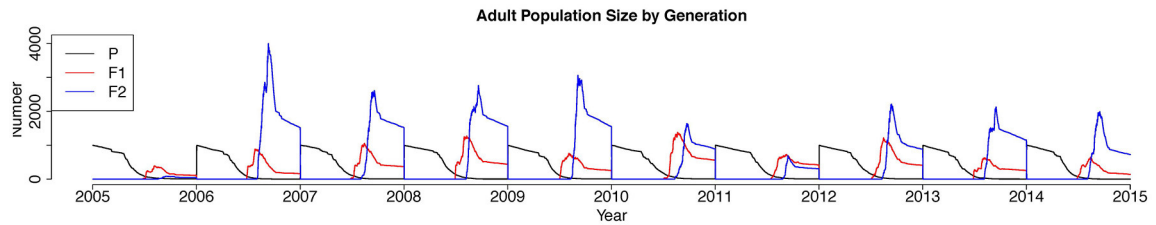

FIGURE 7 | Model predictions of adult population size for Wenatchee, WA. *P* represented parental overwintered adults, which was initialized as 1,000 for each year and for each simulation run.

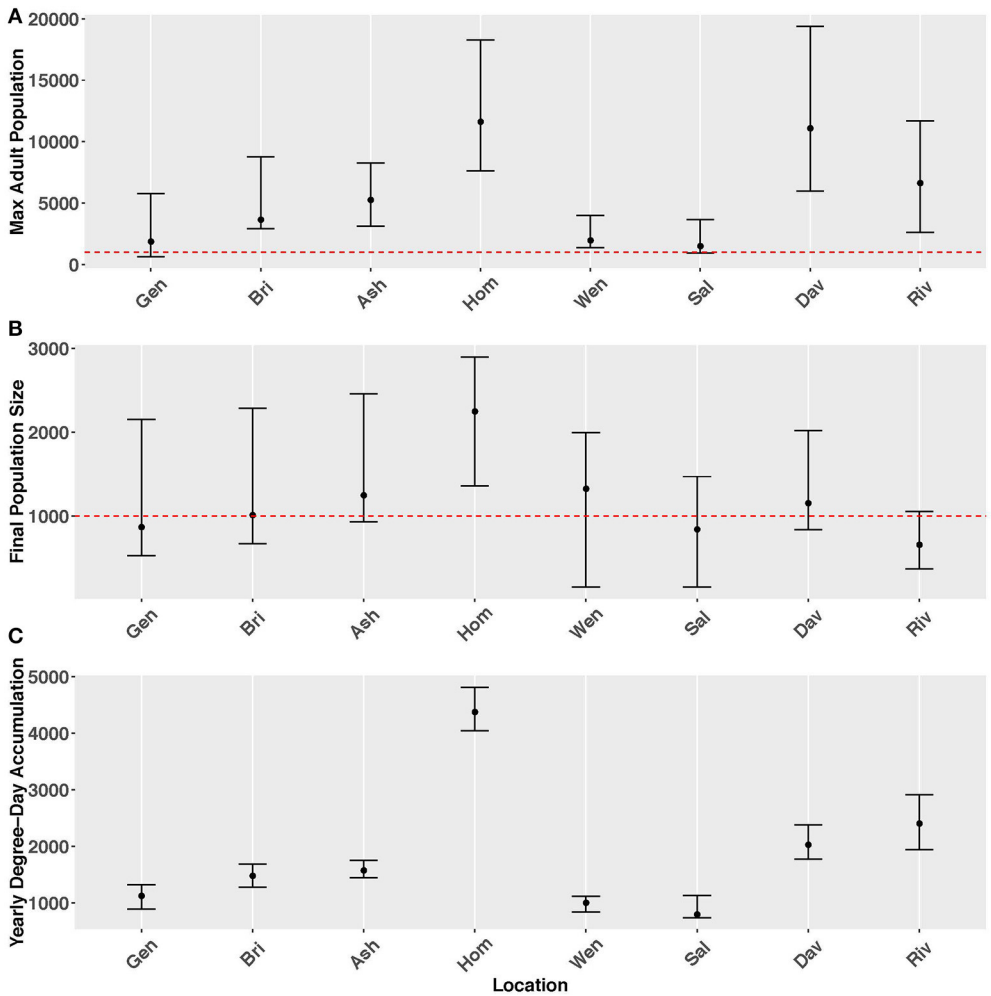

FIGURE 8 | Predicted population sizes (+/− range from all simulations and years) across all geographic locations for **(A)** maximum adult population size, and **(B)** final population size, and **(C)** yearly degree-day accumulation. The error bars represent the standard errors from 100 simulations for the metrics.

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ORIGINAL RESEARCH
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Microclimate Data Improve Predictions of Insect Abundance Models Based on Calibrated Spatiotemporal Temperatures

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A large body of literature has recently recognized the role of microclimates in controlling the physiology and ecology of species, yet the relevance of fine-scale climatic data for modeling species performance and distribution remains a matter of debate. Using a 6-year monitoring of three potato moth species, major crop pests in the tropical Andes, we asked whether the spatiotemporal resolution of temperature data affect the predictions of models of moth performance and distribution. For this, we used three different climatic data sets: (i) the WorldClim dataset (global dataset), (ii) air temperature recorded using data loggers (weather station dataset), and (iii) air crop canopy temperature (microclimate dataset). We developed a statistical procedure to calibrate all datasets to monthly and yearly variation in temperatures, while keeping both spatial and temporal variances (air monthly temperature at 1 km^2 for the WorldClim dataset, air hourly temperature for the weather station, and air minute temperature over 250 m radius disks for the microclimate dataset). Then, we computed pest performances based on these three datasets. Results for temperature ranging from 9 to 11°C revealed discrepancies in the simulation outputs in both survival and development rates depending on the spatiotemporal resolution of the temperature dataset. Temperature and simulated pest performances were then combined into multiple linear regression models to compare predicted vs. field data. We used an additional set of study sites to test the ability of the results of our model to be extrapolated over larger scales. Results showed that the model implemented with microclimatic data best predicted observed pest abundances for our study sites, but was less accurate than the global dataset model when performed at larger scales. Our simulations therefore stress the importance to consider different temperature datasets depending on the issue to be solved in order to accurately predict species abundances. In conclusion, keeping in mind that the mismatch between the size of organisms and the scale at which climate data are collected and modeled remains a key issue, temperature dataset selection should be balanced by the desired output spatiotemporal scale for better predicting pest dynamics and developing efficient pest management strategies.

Keywords: insects, scale, temperature, microclimate, models, agriculture, landscape

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INTRODUCTION

Ectotherms rely on environmental heat sources that permit them to operate at very economical metabolic rates, i.e., low energetic costs (Sears and Angilletta, 2015). Their internal physiological sources of heat are relatively small or quite negligible in controlling body temperature (e.g., plants, small insects; Huey and Stevenson, 1979; Brown et al., 2004; Cossins, 2012). Therefore, ectotherms regulate their body temperature making use of their abiotic environments that is both temporally and spatially heterogeneous. The effect of temperature variability on the physiology and ecology of ectotherms have generally been addressed using data from either coarse-scale climatic models or weather station networks. However, a large body of literature has acknowledged that large-scale climatic data misrepresent the thermal environment of living organisms (Holmes and Dingle, 1965; Angilletta, 2009; Geiger et al., 2009; Buckley et al., 2013; Bennie et al., 2014; Hannah et al., 2014; Kearney et al., 2014), especially for tiny organisms such as insects. Consequently, accurately predicting how small ectotherms respond to climatic variability requires reducing the mismatch between the spatial scales of climatic data and the body size of the organism studied (Potter et al., 2013; Faye et al., 2014).

Ectotherms' responses to temperature are commonly modeled with performance curves (Angilletta, 2009) that describe performance (e.g., survival, growth, fecundity) along a continuous thermal gradient. An important application of performance functions is the construction of population models that simulate insect life-history events, phenology, and distribution under varying environmental conditions over time. A great variety of temperature-based models [e.g., species-distribution models (SDMs, Elith and Leathwick, 2009), cohort-based models (Logan, 1988); individual-based models (Buffoni and Pasquali, 2010); cellular automata (Rebaudo et al., 2011)] have been developed to assess the level of fitness of insect populations across natural and anthropogenic landscapes. Such models are becoming a key component of insect population outbreaks both under current and predicted climatic conditions (Venette et al., 2010).

A key issue of the use of these models is their sensitivity to the spatiotemporal resolution of input temperature datasets (from global to local, annual to hourly). This question has been debated since the birth of SDMs (Guisan and Thuiller, 2005), with some authors suggesting that finer-scaled SDMs provide better predictions (Elith and Leathwick, 2009; Hannah et al., 2014; Storlie et al., 2014) and others that they do not (Guisan et al., 2007; Bennie et al., 2014). Fine-resolution spatial data may be less important for organisms in spatially homogeneous environments or for wide-ranging studies that focus on a general purpose and trends. Also, high temporal resolution data may be less important in environments where diurnal or seasonal variability is limited, at least relative to the environmental tolerances of organisms (Potter et al., 2013).

Traditional agricultural landscapes such as those found in a wide area of the tropical belt, are typically made of a mosaic of small (<1 ha) crop fields at various stages of maturation (Dangles et al., 2008). This creates highly heterogeneous thermal conditions at local scale, resulting in the fact that coarse-scale climate data hardly capture the climatic reality experienced by crop insects (Faye et al., 2014). In such systems, the reliability of pest dynamics models may therefore strongly depend on the resolution of temperature dataset used. To test this hypothesis, we implemented pest performance models with air temperature datasets obtained at three different spatiotemporal resolutions: (i) WorldClim dataset (global dataset), (ii) air hourly temperature at the weather station location (weather station dataset), and (iii) crop canopy temperature data measured every minute at various phenological stages at the field scale (microclimate dataset). We then confronted the outputs of these population dynamics models to field data of crop infestation obtained during a 6-year long monitoring survey. To achieve this goal, the main step of our study were (i) to develop a statistical procedure to calibrate all data sets to monthly and yearly variation in temperatures, (ii) to run and compare the outputs of the three models (global, weather station, and microclimate) in terms of pest performance, and (iii) to compare prediction of the three models with pest performance data obtained in the field.

MATERIALS AND METHODS

Study Sites

The study area was located in the province of Cotopaxi (01°01′36″S, 78°32′16″W), Ecuador, at four sites where pests and temperatures were monitored (**Table 1**). Those sites were chosen along a gradient of elevation (from 2700 to 3300 m.a.s.l.) with two sites at low elevation and two sites at high elevation. They were composed of a mosaic of small fields and pastures, generally smaller than 1 ha (Dangles et al., 2008). Depending on elevation, the main crops were potato (*Solanum tuberosum* L.), broad bean (*Vicia faba* L.), corn (*Zea mays* L.), and alfalfa (*Medicago sativa* L.). Despite of the occurrence of two main seasons in the Ecuadorian Andes (higher temperatures from November to May), temperature amplitude over 1 year is low (Bonebrake and Deutsch, 2012). The intra-annual standard deviation of mean monthly temperature measured at our study sites ranged from 0.54 to 0.66°C. Moreover, temperature variations within a month are comparable to those recorded within a year (intra-month standard deviation of mean daily temperature = 1.07 ± 0.16°C).

Potato Moth Monitoring

At the four study sites, we monitored over 6 years the fluctuations in population levels of three tuber feeding moth species (from 2006 to 2012): *Phthorimaea operculella* Zeller, *Tecia solanivora* Povolny, and *Symmetrischema tangolias* Gyen (Lepidoptera: Gelechiidae). *P. operculella* supposed origin is the mountainous region of South America (Sporleder et al., 2004). It has been reported in more than 90 countries worldwide, mostly in tropical and subtropical potato production areas (Kroschel et al., 2013). *S. tangolias* was first reported in Peru in 1931, and progressed northward to Ecuador (Dangles et al., 2008). *T. solanivora* originated from Guatemala and is currently distributed up to Ecuador (Puillandre et al., 2008). These three species present a major threat to the food security of farmers in Central America and the Northern Andes (Dangles et al., 2009), and

TABLE 1 | Location and characteristics of the four monitored sites in the central Ecuadorian province of Cotopaxi.

Site code	Lowland 1	Lowland 2	Highland 1	Highland 2
Site local name	La Hoya	Anchilivi	Palama Medio	Palama Bajo
Coordinates in decimal degrees	−1.00; −78.57	−1.05; −78.56	−1.00; −78.52	−1.01; −78.53
Elevation (m.a.s.l.)	2713	2727	3280	3152
Number of fields	40	84	74	58
Fields area (ha)	16.8	16.3	22.2	18.0
Average field size (m^2)	4198 ± 5376	1937 ± 1177	2999 ± 1794	3104 ± 2383
Mean temperature from the WorldClim	13.3 ± 0.58	13.71 ± 0.63	10.1 ± 0.52	10.81 ± 0.55
Mean total pest abundance per month	139 ± 91	134 ± 78	86 ± 66	99 ± 57

are found in co-occurrence in Ecuador both in the fields and the storage facilities (Dangles et al., 2008). *T. solanivora* only fed on potato tubers, while *S. tangolias* and *P. operculella* can fed on potato tubers, leaves and stems (Dangles et al., 2008). The three species pupate in the soil near the plants, in leaf remains, in potato storages, or in any suitably sheltered sites, as described in Dangles et al. (2008). They are poikilothermic organisms sensitive to abiotic factors, and are strongly driven by temperature (Dangles et al., 2008; Crespo-Pérez et al., 2015). Although studies have highlighted the importance of precipitations (Foot, 1974; Whiteside, 1980), little influence has been observed on moth abundance in the case of equatorial region (Crespo-Pérez et al., 2015). Under the climatic conditions of the Ecuadorian highlands, tuber moth populations are active all year round and neither diapause nor seasonal rhythms have been reported for these three moth species. Pest monitoring was performed using pheromone traps specific to each potato moth species following the protocol described by Crespo-Pérez et al. (2011) and Dangles et al. (2010). The pheromone traps were collected every 3 weeks during 6 years. The potato moth monitoring at these four sites were devoted to the establishment of predicting models analyzed at the temporal scale.

Additionally, potato moth monitoring was realized in 15 supplementary sites in the Ecuadorian Andes as part of a larger monitoring network (see Dangles et al., 2008 for further details). Those sites were used to compare predicted vs. field abundance of potato moth at the spatial scale, testing the ability of our previously built models to predict pest crop abundances outside of the initial altitudinal range. These additional sites were all located within the central provinces of Ecuador (Bolívar, Chimborazo, Cotopaxi, Tungurahua; representing 19,939 km^2), along an altitudinal range from 2600 to 3600 m. They shared similar characteristics in term of landscape composition and agricultural practices.

Temperature Monitoring

At each study site, we monitored temperature at three spatiotemporal scales, referred as temperature datasets (**Figure 1**).

For the global dataset, we extracted the mean air temperatures over the last 50 years from the WorldClim database (Hijmans et al., 2005), at an available spatial resolution of 30 arc seconds (equivalent to squares of 0.86 km^2 close to equator). Among numerous previous studies, the worldClim dataset has been used to predict the distribution of *P. phthorimaea* (Kroschel et al., 2013), *T. solanivora* (Schaub et al., 2009; Crespo-Pérez et al., 2013), and to relate abundances of *S. tangolias* with temperature (Dangles et al., 2010). This dataset therefore served as a reference for our study case, while being relevant for other species, given its popularity in species distribution models (Elith et al., 2006; Lobo et al., 2010).

For the weather station dataset, we recorded air temperature using loggers (Hobo U23-001-Pro-V2, Onset Computer Corporation, Bourne, USA) at each of the four sites. Temperature data loggers were positioned on a wooden stake at 1.5 m high and then sheltered by white plastic roof to minimize solar radiation heating. The data loggers recorded temperature every half an hour with an accuracy of ±0.21 K over the 0–50°C range together with a resolution of 0.02 K at 25°C, as described by Faye et al. (2014). We then computed the monthly temperatures based on these data to compare the air temperature from the data loggers with the global dataset. This dataset corresponds to commonly used weather stations data.

For the microclimate dataset, we recorded air temperature inside crop canopies using various data loggers for all crop types and phenologies in the 250 m radius disk from the location of the air temperature logger of the weather station dataset (see **Table 1** for more information on the study sites and **Figure 1**). As described in Faye et al. (2014), the data loggers were positioned 5 cm bellow the top of crop canopy to avoid the effect of direct solar radiation. The radius distance of 250 m corresponded to the mean maximum dispersal distance of the pest species considered in this study (Crespo-Pérez et al., 2011). To obtain this dataset, we recorded air temperature and air temperature inside canopy every minute for 15 days in October 2011 (see Faye et al., 2014 for details), resulting in 162 independent measurements for our four study sites. This dataset corresponds to the closest temperature information to temperature actually experienced by pests, and is therefore referred as the microclimate dataset.

Consequently we used a global temperature dataset from the WorldClim database at a 30-arc seconds spatial resolution, a local weather station dataset at four study points, and a microclimate dataset integrating all canopy temperatures in a landscape disk of 250 m radius. For convenience, in the rest of this paper we used the terms "*WorldClim*" dataset,

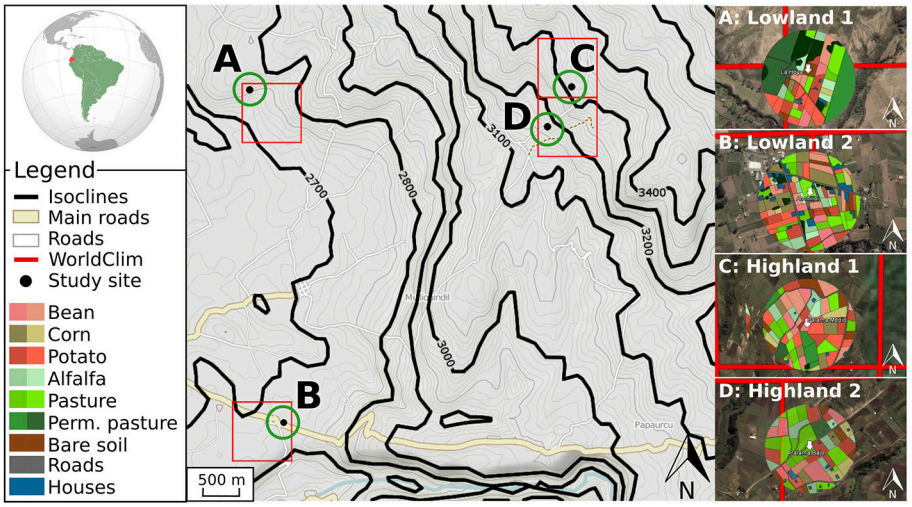

FIGURE 1 | Schematic representation of the three temperature datasets. The global dataset is shown through red lines defining squares of 0.86 km^2 corresponding to the *WorldClim* database. The *Weather stations* dataset is represented with black points **(A–D)** corresponding to the coordinates of the data loggers. The *Microclimate* dataset is schematized with green circles and colored disks (right side of the figure) with a different color for each crop and phenological stages, corresponding to the air temperature inside canopy.

"*Weather stations*" dataset, and "*Microclimate*" dataset when referring to the first, second, and third temperature datasets, respectively.

Temperature Dataset Standardization

To compare the effect of the three temperature datasets on modeled pest performances and abundances, we used the *WorldClim* dataset as a reference. The raw *Weather stations* dataset corresponded to temperatures collected during 3 years, and the raw *Microclimate* dataset to temperature collected during 15 days in all fields. Consequently, we transformed and extrapolated the raw temperatures of the *Weather stations* and *Microclimate* datasets to fit the temporal resolution of the *WorldClim* dataset (i.e., monthly temperatures), but kept the spatial resolution of these datasets, i.e., at one punctual monitoring site for the *Weather stations* dataset and the field-based 250-m landscape radius for the *Microclimate* dataset. This process resulted in a single temperature value per month in the case of the *WorldClim* dataset, a set of temperature values per month in the case of the *Weather stations* dataset (corresponding to the monthly variance observed among years), and a set of temperature values per month in the case of the *Microclimate* dataset (corresponding to both the spatial variance observed in the landscape and the monthly variance observed among years).

The *Weather stations* dataset was transformed by aggregating the 30 min temperatures for each month. Then we decomposed the monthly time series to obtain the seasonal variation, the trend and the random component using the R package "stats" (R Core Team, 2015). We subtracted the trend to the dataset in order to avoid the effect of the monitored years on average temperature, and checked the cross-correlation between the seasonal variation in the *WorldClim* dataset and the *Weather stations* dataset (significant cross-correlation at lag 0 with ccf = 0.825, 0.862, 0.901, and 0.907 for the four monitored sites, respectively). According to the cross-correlation, we assumed the *WorldClim* dataset seasonality as a reference of seasonality, so that we kept only the random component of the *Weather stations* dataset. We then modeled the distribution of the *Weather stations* dataset random component to simulate new monthly temperatures over multiple years, representative of temperature monitored in the field. The obtained dataset was therefore representative of the temperature variance for each month between years.

For the *Microclimate* dataset, we first mapped the field contours in a radius of 250 meters using ArcGIS 10.01 (ESRI, Redlands, USA). As crop phenologies and rotations have been shown to affect microclimates in this region (Faye et al., 2014), we built a model representing the crop rotation (see Table SI A.1 for classical crop rotations in the study area), using the GAMA modeling and simulation development environment for building spatially explicit agent-based simulations (Grignard et al., 2013; https://github.com/gama-platform/gama/wiki). The initial stage of each crop was chosen randomly in the crop rotations, according to the proportions of observed phenological stages in our study area. Using air temperature datasets and canopy temperature datasets, we built another model explaining air temperature inside the canopy as a function of air temperature (linear model). Assuming that the relationship between air temperature and air temperature inside canopy was constant over the year, for each air temperature from the *Weather station* dataset, we computed monthly air temperatures inside canopy for each crop and over multiple years. This model was therefore representative of the temperature variance at the spatial scale (all fields within a radius of 250 m), and at the temporal scale (temperature variance for each month between years).

Crop Pest Performance Modeling

Pest performance models were based on the temperature-dependent performance curves of the three potato moths in terms of survival rate, developmental rate, and fecundity (in number of eggs per female). Temperature dependent survival and developmental rates were based on the non-linear thermodynamic model developed by Sharpe and DeMichele (1977) and modified by Schoolfield et al. (1981) (Equation 1), and fecundity was based on the gamma function (Equation 2) as described and fitted in previous studies on these crop pests (Crespo-Pérez et al., 2011; Rebaudo and Dangles, 2011; Rebaudo et al., 2011). These models were chosen for their wide range of application among arthropods (e.g., Fand et al., 2015; Ramalho et al., 2015); and especially among Lepidoptera (e.g., Kim et al., 2001; Khadioli et al., 2014).

$$D(T) = \frac{\frac{dT}{298.16} exp\left[\frac{e}{R}\left(\frac{1}{198.16} - \frac{1}{T}\right)\right]}{1 + exp\left[\frac{f}{R}\left(\frac{1}{g} - \frac{1}{T}\right)\right] + exp\left[\frac{h}{R}\left(\frac{1}{i} - \frac{1}{T}\right)\right]} \quad (1)$$

with T the temperature in Kelvin, $R = 1.987$, and d, e, f, g, h, and i estimated parameters from previous studies using least square minimization techniques.

$$F(T) = o + p * exp\left(-\frac{T-q}{r}\right)\left(\frac{\frac{T-q}{r} + s - 1}{s-1}\right)^{s-1} \quad (2)$$

with T the temperature in Celsius, and o, p, q, r, and s estimated parameters from previous studies using least square minimization techniques.

Data Analysis

Comparison of Modeled Performances among Species

We used the standardized *WorldClim*, *Weather stations*, and *Microclimate* temperature datasets as inputs for pest performance models based on performance curves described above. The *WorldClim* and *Weather stations* datasets served as reference datasets to compare performances for each pest. We then computed the relative difference in performances between each dataset as a function of the average temperature for the *WorldClim* dataset.

Comparison of Predicted vs. Field Abundance of Potato Moth

Temporal analysis

We used the three standardized temperature datasets (see Section Temperature Dataset Standardization) to simulate potato moth abundances for each month of a given year. Then, our 6-year potato moth abundance data were transformed to obtain monthly abundances over the year with 6 repetitions for each month. We then confronted monthly potato moth abundances as a function of monthly temperatures and associated performances as presented below.

To relate species performance simulated with the three temperature datasets with the pest abundances, we first calculated mean and quartile values of each temperature dataset and each performance. We then used multiple linear regressions with a stepwise analysis to minimize the AIC and find the best model explaining pest abundances for each temperature dataset and evaluate the relevance of the *Microclimate* dataset (Equation 3).

$$N \sim S() + D() + F() + temp \quad (3)$$

with N the potato moth abundance, *S()* the survival rate, *D()* the developmental rate, *F()* the fecundity, and *temp* the temperature. For parameters *S()*, *D()*, *F()*, and *temp*, we considered the mean and quartiles values in the stepwise analysis.

Spatial analysis

We then tested these models with another set of potato moth monitoring data composed of 15 sites spread out over a gradient of elevation from 2600 to 3600 m in Ecuador (see Section Potato Moth Monitoring). This spatial validation aimed at validating to which spatial extent the models built on the basis of our four monitored sites could be extrapolated to this altitudinal range, even in the absence of *Weather stations* and *Microclimate* datasets available for those sites.

RESULTS

Temperature Models

The *Weather stations* dataset random component followed a Gaussian distribution (Shapiro–Wilk tests, W = 0.993, 0.986, 0.992, 0.977 and p-value = 0.999, 0.970, 0.998, 0.846 for the four sites, respectively). We therefore simulated the *Weather stations* dataset using the *WorldClim* seasonality component and a Gaussian distribution for the random component with parameters fitted for each of the study sites. The resulting model allowed simulating multiple years with the associated variance in temperatures for the four sites (**Figure 2**).

For the *Microclimate* dataset, the relationship between air temperature and air crop canopy temperature followed a linear model (**Table 2**). The model showed a buffering effect of plant canopy over air temperature, with warmer temperatures inside canopy at low air temperature (sites on **Figures 2C,D**), and colder temperature at warm air temperature (see sites on **Figures 2A,B**). The transformed *Microclimate* dataset fitted a normal distribution (Shapiro–Wilk normality test, W = 0.99, $p = 1.439e\text{-}13$), which can be observed in boxplots of **Figure 2**.

Discrepancies among Predictions of Species Performance

Outputs of pest performance models implemented with different temperature datasets (*Weather stations* and *Microclimate* vs. *WorldClim*) were similar for some study sites (e.g., survival rate in **Figures 3A,B**) but differed for others (e.g., survival rate in **Figures 3C,D**). For example, at site C, we observed that survival rates predicted using the *WorldClim* dataset were close to zero for the months of July and August, while both *Weather stations* and *Microclimate* models predicted positive survival rates, up to 0.2 on the third quartile (**Figure 3C**). Moreover, while the *WorldClim* and the *Weather stations* datasets resulted in similar performance means, the buffering effect of the *Microclimate*

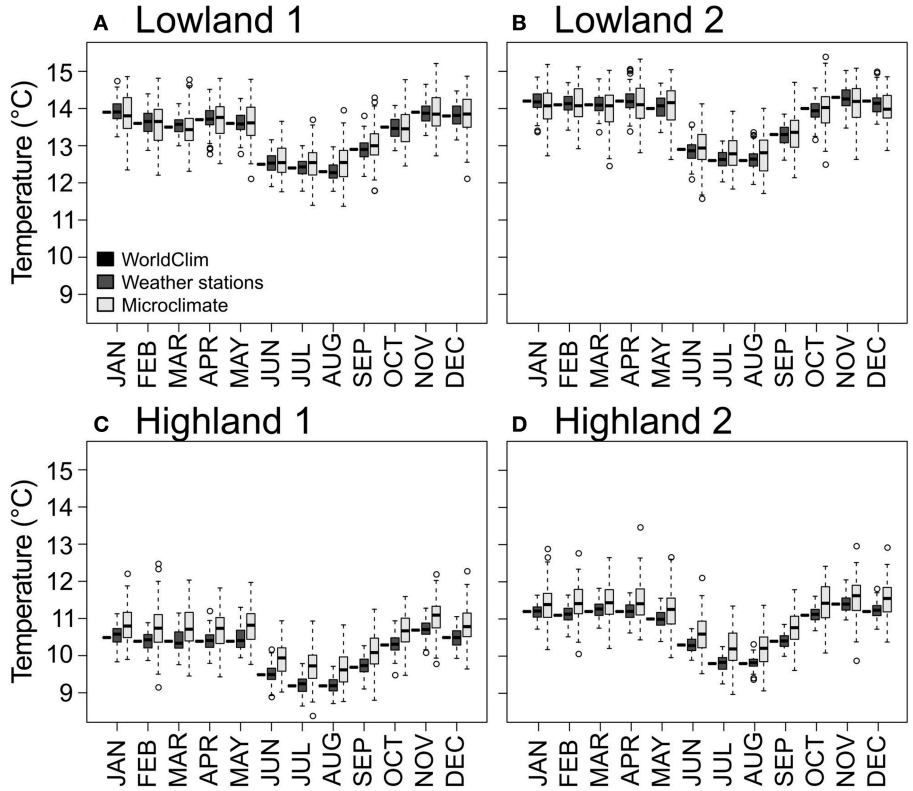

FIGURE 2 | Standardized temperatures for each month and each dataset. The *WorldClim* dataset is represented as black horizontal bars. The *Weather stations* and *Microclimate* datasets are represented as dark gray and light gray boxplots, respectively. Panels **(A–D)** for the four study sites.

dataset predicted higher performances, either in terms of survival rate, developmental rate, or fecundity.

At low temperatures (9–11°C), performance simulations revealed that the use of the *WorldClim* dataset tended to underestimate survival rate in comparison to the use of the *Microclimate* dataset (up to +100%), while the use of the *WorldClim* dataset led to both under- and overestimate the survival rate in comparison to the use of the *Weather stations* dataset (between −300 and +100%, **Figure 4**). At warmer temperatures (12–15°C), performance simulations based on the three datasets revealed almost identical pest survival rates. Simulations of developmental rate and fecundity showed less variation when estimated with the three temperature datasets.

Model Reliability to Predict Pest Field Abundance

Pest abundances and their standard deviations as a function of months are represented as bar plots in the top of **Figure 3**. For the models presented in **Table 3** based on the three temperature datasets, we found no significant differences between observed and predicted abundances of potato moth (Student tests, $p > 0.43$, **Figure 5**). The lowest AIC and highest r-squared values were found for the model based on the *Microclimate* dataset (**Figure 5**), indicating a better accuracy of this model over the two other temperature datasets (**Table 3**). The shape of the boxplots in **Figure 5** also indicates that predictions based on the *WorldClim* and *Weather stations* datasets tended to smooth abundances between months over the year (low variance compared to the observed abundances) while the *Microclimate* dataset better represents intra-annual variation in potato moth abundances.

To assess the robustness of our findings at a larger spatial scale, we compared observed and predicted potato moth abundances in 15 sites spanning a range of elevations in Ecuador. We found an average difference of 56, 74, and 80% between observed and predicted abundances for the models based on the *WorldClim*, *Weather stations,* and *Microclimate* datasets, respectively, indicating that the *WorldClim* dataset best predicted potato moth abundances at larger spatial extent. We also found a significant effect of elevation over the goodness of fit (linear models with significant positive slopes, $p < 0.05$, $r^2 = 0.16$, 0.17, and 0.09 for the *WorldClim*, *Weather stations,* and *Microclimate* datasets, respectively), with a higher difference between observed and predicted abundances as the elevation increases.

DISCUSSION

Many organisms take advantage of different habitats to perform their life cycle. For example, arthropods such as moths spend most of their cycle as a larvae or pupae located in the plant or soil layers, where temperature experienced is in the range

TABLE 2 | Relation between air temperature and air temperature inside canopy for all crops at two phenological stages and two altitudinal ranges [low (A) and high (B) elevation].

Altitudinal range	Crop	Phenological stage	Linear model	*p*-value	R-squared
A	alfalfa	1	Tplt = 2.6155 + 0.8048 * Tair	***	0.3741
A	alfalfa	2	Tplt = 6.2248 + 0.5754 * Tair	***	0.215
A	bean	1	Tplt = 3.4655 + 0.6863 * Tair	***	0.9657
A	bean	2	Tplt = 0.05918 + 0.9552 * Tair	***	0.4966
A	corn	1	Tplt = −3.3529 + 1.2681 * Tair	***	0.8206
A	corn	2	Tplt = 2.8026 + 0.7680 * Tair	***	0.8563
A	pasture	1	Tplt = −0.2593 + 1.1113 * Tair	***	0.6318
A	pasture	2	Tplt = 5.9341 + 0.5464 * Tair	***	0.5804
A	potato	1	Tplt = −3.0608 + 1.2029 * Tair	***	0.7089
A	potato	2	Tplt = 3.8663 + 0.7018 * Tair	***	0.4248
A	bare soil	–	–	–	–
B	alfalfa	1	Tplt = 0.8681 + 1.0350 * Tair	***	0.4932
B	alfalfa	2	Tplt = 7.38339 + 0.31449 * Tair	***	0.3017
B	bean	1	Tplt = 7.03727 + 0.35023 * Tair	***	0.674
B	bean	2	Tplt = 0.87834 + 0.88096 * Tair	***	0.9315
B	corn	1	Tplt = 1.0665 + 0.9809 * Tair	***	0.8702
B	corn	2	Tplt = 2.2445 + 0.7935 * Tair	**	0.946
B	pasture	1	Tplt = −2.1935 + 1.3620 * Tair	***	0.5521
B	pasture	2	Tplt = 3.3894 + 0.6443 * Tair	***	0.3717
B	potato	1	Tplt = 0.41817 + 0.96059 * Tair	***	0.9559
B	potato	2	Tplt = 3.50316 + 0.71010 * Tair	***	0.4934
B	bare soil	–	–	–	–

*No linear models were computed for the fields corresponding to bare soil, assuming that difference in air temperatures in the first 2 m above ground level was negligible (Kearney et al., 2014), and that the air temperature was representative of the temperature experienced by insects. P-values below 0.005 are represented with "***" and p-values below 0.05 with "**".*

of their optimums (Scherrer and Körner, 2011; Suggitt et al., 2011; Scheffers et al., 2014), while others actively modify local conditions [e.g., aggregations in colonies (Danks, 2002); thermoregulation (Willott, 1997; Pincebourde and Casas, 2006)]. In our case, results showed that the model based on the microclimate temperature dataset predicted the best result, which is, in that sense, rather intuitive. Pests likely take advantage of the landscape they live in for finding food and seeking optimum temperatures, but also for finding shelter from lethal conditions (Hart and Resh, 1980; Füreder, 1999), and thereby reducing their exposure to climate extremes (Scheffers et al., 2014). The Ecuadorian Andes are characterized by highly heterogeneous agricultural landscapes, which may offer better chances of finding optimal thermal habitats within a relatively low distance (Faye et al., 2014). It is therefore sound that, at the local scale, predicting abundances on the basis of temperature datasets that ignore this heterogeneity proved to be poorly effective. The species considered in this study are highly sensitive to temperature variations (Dangles et al., 2008, 2009; Kroschel et al., 2013), which placed them as specialist in the thermosensibility scale. This is mostly the case of all small ectotherms which are thermoconformers, a major constraining factor in their dispersion (Overgaard et al., 2014). Our findings thus applied to similar organisms sensitive to local conditions (Woods et al., 2015), while other species may be modeled regardless of the temperature spatiotemporal resolution. Moreover, for the species considered in this study, part of the cycle occurs in the soil layer (Dangles et al., 2008), which was not monitored. Knowledge of the temperature experienced by species in the soil could have explained in better details the observe abundances, as temperature in the soil layer differs from air temperature (Parton and Logan, 1981), in relation to its composition, exposure, and humidity. This implies that temperature dataset should be based on the species considered (body length and dispersal capacity; Potter et al., 2013; Hannah et al., 2014) and the spatiotemporal heterogeneity of temperature in the landscape (spatiotemporal heterogeneity of the climatic conditions in which the study organism evolved along its life cycle).

However, results revealed that the model based on the *Microclimate* dataset failed to explain abundances outside of the range of the four study sites, and that sites located at higher elevations were more prone to mis-predicted abundances. This limitation may be due either to the model itself that could be sensitive to changes in elevation, or because the extrapolation of the temperatures from our monitored sites does not apply to this case. Therefore, two challenges should be overcome in future studies: (i) the lack of microclimate dataset available at large scale, as already acknowledged in numerous studies and despite promising directions offered by downscaling models (Potter et al., 2013; Kearney et al., 2014) or recent advances in microclimatic data collection (Faye et al., 2015), and (ii) the choice of scale at which a model can be applied, which is a recurrent question in species distribution modeling (Wiens, 2002; Guisan and Thuiller, 2005). Our study

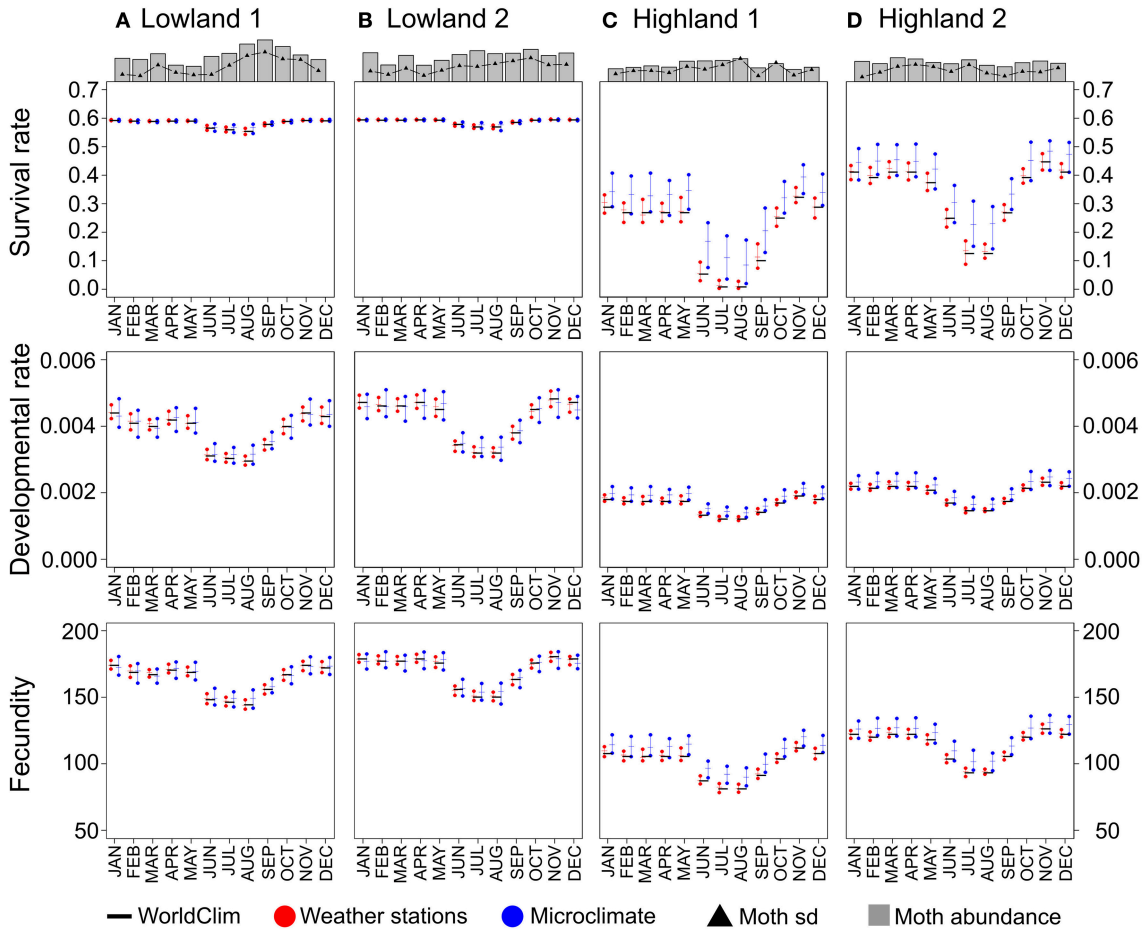

FIGURE 3 | Pest abundances and performances for each site based on the three temperature datasets. Pest abundances represent the mean sum of abundances for the three potato moth species. Black triangles display the standard deviations for each month. Performances were computed for each site using the mean values for the three potato moth species. *WorldClim* dataset based performances are represented as black horizontal bars. *Weather stations* and *Microclimate* datasets based performances are represented as red and blue points for the first and third quartiles, and as horizontal bars for the median. Panels **(A–D)** for the four study sites.

TABLE 3 | Multiple linear regression models explaining the potato moth abundances for each temperature dataset.

Dataset	Model	AIC	R-squared
WorldClim	N ~ S() + D() + F() + temp	314	0.51
Weather stations	N ~ F() + temp + q3S() + q3D() + q3F() + q3temp	311	0.57
Microclimate	N ~ S() + F() + temp + q1D() + q1F() + q1temp + q3F() + q3temp	307	0.64

N represents potato moth abundances. S(), D(), F(), temp represent the mean survival rate, developmental rate, fecundity, and temperature, respectively. q1 and q3 represent the first and third quartiles. The AIC corresponds to the lowest value computed from the stepwise analysis.

supports that in the absence of microclimate dataset, global climate models are best suited to predict species abundances at large scale (and low resolution), while microclimate datasets best predict abundances at fine scale (and high resolution), as highlighted in other studies on the effect of spatial resolution on species distribution (Gillingham et al., 2012). Regarding the specific effect of elevation on predicted abundances, our results highlight the complexity of insect responses to altitudinal gradients (McCoy, 1990; Lomolino, 2001; Hodkinson, 2005; Sundqvist et al., 2013), impeding our models to accurately predict potato moth abundances at higher elevations. The change in the composition of potato moth species as a function of elevation (Dangles et al., 2008, 2009), and the difference in farmer practices (Rebaudo and Dangles, 2011), may add another layer of complexity to the complex agro-ecosystem in place.

In addition, the models used in this study were built on the basis of laboratory experiments performed under controlled climatic conditions in rearing units (Crespo-Pérez et al., 2011;

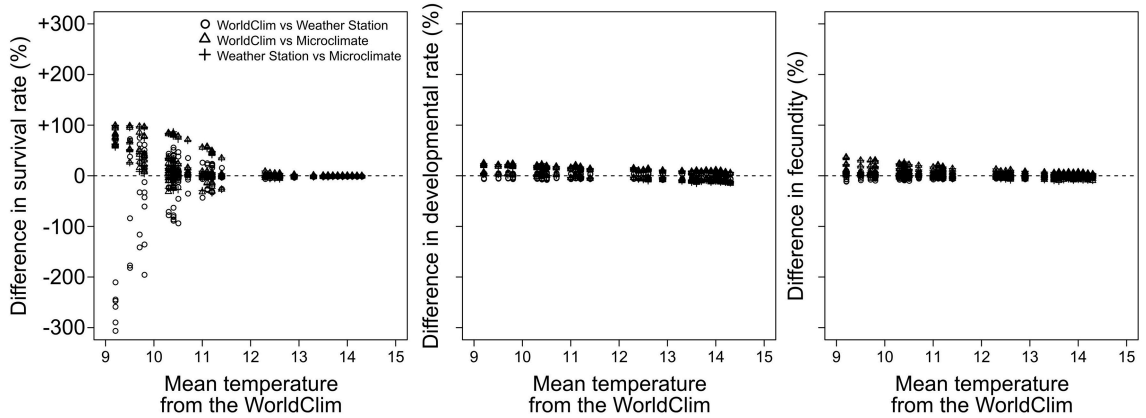

FIGURE 4 | Comparison between pest performances computed with the different temperature datasets as a function of the WorldClim temperature. For each performance, circles, triangles, and plus signs represent the difference between the *WorldClim* and the *Weather stations*, the *WorldClim* and the *Microclimate*, and the *Weather stations* and the *Microclimate* datasets, respectively.

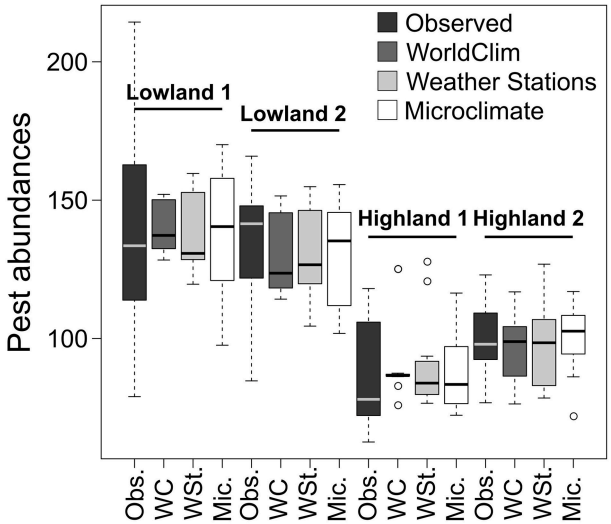

FIGURE 5 | Observed and predicted abundances computed with the different temperature datasets for the four studied sites. Pest abundances are represented as boxplots and correspond to all pest abundances per month.

Rebaudo and Dangles, 2011; Rebaudo et al., 2011). If their applicability could predict presence absence successfully at regional scales with coarse resolution (Crespo-Pérez et al., 2011), increasing the resolution of temperature dataset could logically yield more accurate results. Consequently, temperature datasets should be employed with models built on comparable resolutions (i.e., in the model range of applicability, Rykiel, 1996). Moreover, the type of demographic models to be used will obviously influence the resulting predictions: models making uses of extreme temperatures, known to constrain ectotherms (Hoffmann et al., 2013), or night-time temperatures (Zhao et al., 2014), may predict species abundances more accurately at higher temporal temperature scales, while niche-based models may be better suited for large scales (Thuiller et al., 2005). In addition, our models, based on multiple linear regressions, were designed to correlate temperature and associated performances with pest abundances, and extra-principles such as carrying capacity, pest dispersion, farmers' practices, and Allee effects were not addressed. We fed our models with monthly temperatures using worldClim as a reference dataset. However, diurnal temperature fluctuations are known to influence insect behavior (Taylor, 1963), physiology (Lambrechts et al., 2011), and development (Hagstrum and Milliken, 1991) and may be further considered to improve the predictions of our models. While these could be limitations in order to predict dynamic trajectories, we focused on predicting pest abundances at a given month, compared to pest abundances recorded for this given month over multiple years. This strategy was aimed to diminish the effect of these time-dependent variables, even if reducing the accuracy of our results.

In conclusion, this study supports that in the absence of microclimate dataset, global climate models are best suited to predict species abundances at large scales (and low resolution), while microclimate datasets best predict abundances at fine scales (and high resolution). Therefore, this study stresses the importance to consider different temperature datasets depending on the issue to be addressed. The first point to consider is the species thermosensibility: if small ectotherms would be sensitive to local conditions, most endotherms, less sensitive, would be accurately modeled without the need for microclimate datasets. In the first case, availability of microclimate datasets still represents a challenge to overcome. Second, and jointly with species thermosensibility, the landscape heterogeneity should be taken into account: homogeneous landscapes are likely to share common temperatures at different resolutions. Third, we should consider the accuracy needed to accurately answer the model question: presence absence models at a coarse resolution would require less temperature information than fine-scale abundances models. Based on the case of three moth pests in the Ecuadorian Andes, our study brings new insights into spatiotemporal temperature choice ongoing debate (Austin and Van Niel, 2011; Deblauwe et al., 2016).

AUTHOR CONTRIBUTIONS

FR, EF, and OD contributed to the design of the work, the acquisition, analysis, and interpretation of data, the drafting, and revising. All authors have approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00139

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Looking Beyond the Large Scale Effects of Global Change: Local Phenologies Can Result in Critical Heterogeneity in the Pine Processionary Moth

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SPECIES RESPONSE TO CLIMATE CHANGE: WHY PHENOLOGY AND DISTRIBUTION CAN BE INTERRELATED

Global temperature has increased by 0.85°C between 1880 and 2012, with an acceleration during the last decades and many extreme weather events since about 1950 (IPCC, 2014). A consequence already observed is the change of species distributions. Thermal requirements and constraints are key factors in shaping realized distributions, and climate warming may move or remove these distributional barriers (Walther et al., 2002; Chen et al., 2011). Another consequence of climate warming is a phenology shift for many species (Parmesan, 2006). In plants and ectotherms, shifts in lifecycle timing can result from the direct relationship between temperature and developmental rate (Liu et al., 1995; Chuine et al., 2003). However, phenology is likely driven by a combination of both short-term plastic responses and long-term evolutionary responses to environmental variation (Chuine, 2010; Briscoe et al., 2012).

Consequences of climate change on phenology have often been documented by the study of single life stages (Briscoe et al., 2012). Altered lifecycle timing not only changes climatic conditions undergone by a given stage but can also have cascading effects on subsequent stages. Phenological responses to climate change are thus limited by tradeoffs across the lifecycle, as stages are not independent and often have contrasting thermal sensitivities and requirements (Briscoe et al., 2012). Phenology shifts have been largely studied in plants, showing the role of temporal optimization to cope with several stage-specific constraints (Chuine, 2010). In insects, the overall phenological answer depends on similar complex interactions among life stages with antagonistic or non-additive effects (Briscoe et al., 2012). In turn, these tradeoffs necessarily differ across the distribution due to spatial heterogeneity in climatic conditions and inter-population differentiation. Yet, the relationship between phenology and range shifts has rarely been investigated. Its study requires long time-series of phenology and large-scale surveys of distribution over time, but such historical datasets are rare.

The pine processionary moth (PPM), *Thaumetopoea pityocampa*, is a well-documented Lepidopteran insect which extends its distribution northwards and to higher elevations in Europe as a response to attenuated cold constraint since the 1990s (Roques et al., 2015). This range expansion is acknowledged to be one of the few responses to climate change with direct causal relationship demonstrated (Rosenzweig et al., 2007). For this species, historical data are available not only on distribution at continental scale, but also on phenology and its variation within and among

regions (Géri, 1980; Abgrall, 2001; Roques, 2015). Here, we discuss how climate change could alter phenology in this emblematic species, and how climate spatial heterogeneity interacts with phenology, making the mechanism of range expansion more complex than initially thought.

PINE PROCESSIONARY MOTH, AN INSECT UNDER MULTIPLE THERMAL CONSTRAINTS

PPM is a capital breeder achieving one generation per year (except in case of extended diapause), with winter larval development (Battisti et al., 2015). Eggs are laid in summer and hatching begins approximately 1 month later. Larvae gregariously build a silk tent in pine trees, continue development during winter, and make a procession from their host tree to pupate in the soil in late winter or early spring. After a pupal diapause with flexible duration, adults emerge in summer. Most emergences occur on a short duration (flight peaks) regarding the whole flight period. Because of a short adult lifespan, the mating success depends on synchronous emergences (Démolin, 1969a).

Following Huchon and Démolin (1970), phenology variability is mainly limited by three stage-specific constraints: (a) first instar larvae (L1) are vulnerable to high summer temperatures, (b) second instar larvae (L2) are vulnerable to first autumnal frosts, (c) late instars larvae (L3-L5) are vulnerable to minimal temperatures during autumn and winter, a factor identified as limiting PPM range expansion (Battisti et al., 2005).

PPM phenology was recorded in the 1970s in various French regions (Abgrall, 2001) by monitoring pupation processions and adult flights. Additionally to elevation, latitudinal (South/North) and oceanic-continental (West/East) gradients shape climate variability in France. Here we consider four regions with differentiated PPM phenologies (Abgrall, 2001) and representative of the main French climates (Boutte, 2014; **Figure 1**).

HOW SPATIAL HETEROGENEITY IN THERMAL CONSTRAINTS MAY DRIVE LOCAL PHENOLOGIES

(a) First Instar Larvae are Vulnerable to High Summer Temperatures

Following Démolin (1969b), temperatures above 32°C could perturb physiological processes in eggs and/or larvae, and cause epizootics. He considered that these effects would occur where monthly mean of maximal temperatures are above 25°C. Recent studies showed that eggs from a northern French population are not negatively affected by such temperatures (Robinet et al., 2013), while survival of L1 and L2 from Portuguese populations are negatively affected by temperatures of 36°C and 40°C, respectively (Santos et al., 2011). In France, such high temperatures are the most likely under Mediterranean climate (**Figure 1D**). However, in this area, heat-vulnerable larvae mostly hatch after the highest summer temperatures because flight peaks are late (**Figure 1D**), and can thus experience more suitable temperatures (never reaching 32°C after late August; Démolin, 1969b).

(b) Second Instar Larvae are Vulnerable to First Autumnal Frosts

According to Huchon and Démolin (1970), early instar larvae may be particularly cold-sensitive and must hatch early enough to allow molting into at least the third instar to better resist first cold snaps. In France, early frosts (September) are the most likely in the north-eastern area (**Figure 1B**) but larvae can reach the third instar before the frosts as a consequence of early flight peaks (early July). Flight peaks occur later (mid-late July) in north-western area but cold snaps also appear later (October) due to oceanic influence (**Figure 1A**). The absence of early cold snaps before November in Mediterranean climate allows the development of larvae even following late flights (**Figure 1D**).

(c) Late Instars Larvae are Vulnerable to Minimal Temperatures During Autumn and Winter

Huchon and Démolin (1970) reported the role of minimal temperatures in January-February associated with solar radiation on PPM distribution and lifecycle. Different lethal temperature thresholds have been reported (less than −12°C, Huchon and Démolin, 1970; −17°C, Hoch et al., 2009) but all appear low enough to withstand usual winters in the areas considered. Instead, the main detrimental effects of cold are chill injury due to cumulative negative temperatures (Hoch et al., 2009) and inability to reach the foraging thresholds (Battisti et al., 2005). Cold temperatures in northern areas delay the beginning of processions (**Figures 1A,B**) whereas milder winters in southern areas allow faster larval development and earlier processions (**Figures 1C,D**).

(d) Larval Vulnerabilities May Determine Adult Flight Timing

Factors triggering the end of pupal diapause, and thus adult emergences and local synchrony, are unknown. Nevertheless, the duration of pupal diapause cannot be a simple by product of pupal procession time and climate experienced by the pupae. Indeed, historical data show lower variability for flight periods than procession periods within regions (Abgrall, 2001; **Figure 1**). Moreover, the pupal diapause lasts longer in the South than in the North, despite earlier pupations and warmer climates (Roques and Battisti, 2015; **Figure 1**). This contrasts with typical relationship between temperature and growth rate in ectotherms, thereby suggesting a synchronizing mechanism. To date, there is no evidence that PPM adults are subjected to direct climatic constraints either. We suppose that adult timing is instead indirectly constrained by the aforementioned vulnerabilities of the offspring: hot summers promote late flight peaks and delayed hatching, whereas cold autumns promote early flight peaks and early hatching.

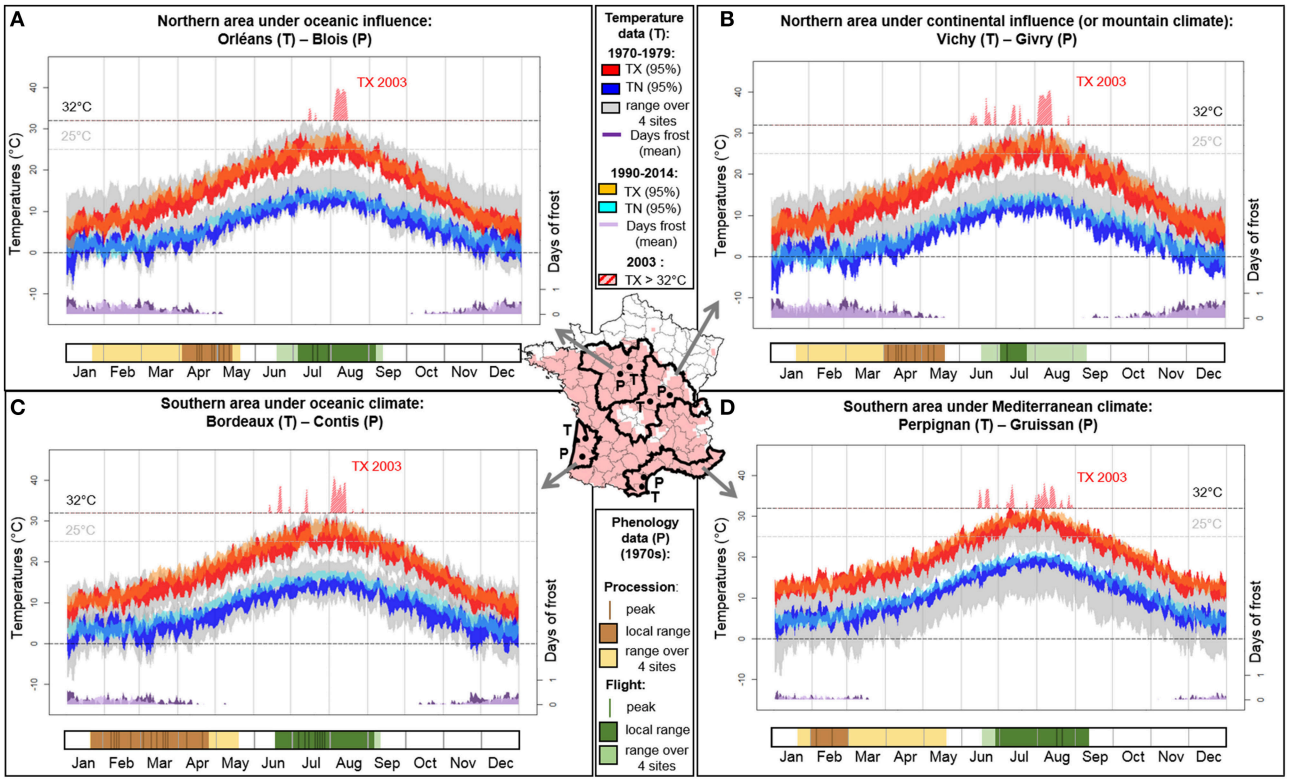

FIGURE 1 | Climate (upper part of each panel; data from http://eca.knmi.nl, Klein Tank et al., 2002) and PPM phenology (procession and flight dates; bottom part of each panel; derived from Abgrall, 2001) in four bioclimatic regions in France. (A) northern area under oceanic influence, **(B)** northern area under continental influence (or mountain climate), **(C)** southern area under oceanic influence, and **(D)** southern area under Mediterranean climate. Hatching occurs 1 month after flights, and L1, L2, and later larval instars until pupation procession are respectively constrained by maximal temperatures in summer, first frosts, and minimal temperatures in winter. Late flight prevents exposure of the L1 progeny to high temperatures (Mediterranean climate) while early flight prevents exposure to early cold snaps (climate with continental influence). Pupation processions are late when winters are cold because of slowed larval development (northern areas) and early when winters are mild (southern areas). Under lower climate constraints (south-western area), flight and procession dates are more widely distributed over time. With climate warming and more frequent heatwaves, PPM phenology may change and its overall distributional response remains difficult to predict despite current beneficial effects on winter larval survival. Daily temperatures (95% confidence interval for TX, daily maximal temperatures; TN, daily minimal temperatures) and mean number of days of frost are shown for 1970–1979 and 1990–2014 (corresponding to the PPM range expansion period; Robinet et al., 2014). Biological thresholds of 0, 25, and 32°C are given for reference. Maximal temperature overreaching 32°C during the 2003 heatwave is represented in red crosshatched. The map gives the PPM distribution in winter 2010–2011 (in light red) in France and the location of the sites where phenology (P) was recorded and where temperature data (T) were retrieved within each of the four bioclimatic zones (see Boutte, 2014, for the definition of these bioclimatic zones).

HOW LOCAL CLIMATES AND PHENOLOGIES LEAD TO HETEROGENEOUS ANSWERS TO CLIMATIC ANOMALIES

Extreme weather events can occur with high spatial heterogeneity (Seneviratne et al., 2012), and increased temperature fluctuations can affect species fitness (Williams et al., 2012). In France, maximal temperatures during the 2003 summer heatwave were 8–13°C higher than the mean over 1990–2014 (**Figures 1A–D**). While French populations collapsed in the two northern areas following this heatwave (Bouhot-Delduc, 2005), an unprecedented altitudinal expansion was observed in the Italian Alps due to unusually numerous nights above the flight threshold (14°C) during this summer, elicitating female flight (Battisti et al., 2006). Although, early-summer flight peaks in these three regions led to hatching during the thermal peaks, L1 experienced suitable temperatures in the Italian Alps (rarely reaching 32°C, Andrea Battisti, Pers. Com.) while they faced lethal conditions in northern French areas (40°C, **Figures 1A–B**).

Another component of heterogeneous responses to climate anomalies is differentiated phenologies. Despite similarly high temperatures, French populations did not crash in areas where late flight peaks occur (**Figure 1C**) or prevail (**Figure 1D**), thus allowing hatching of vulnerable L1 after the constraint. This heatwave illustrates how the fate of local populations can be triggered by (1) spatial heterogenity of large-scale climatic events, but also (2) phenology and differential thermal sensitivity among life stages and populations.

DISCUSSION

Climate change between 1970–1979 and 1990–2014 resulted in an average winter warming in the PPM bioclimatic zones

considered (October to March: +0.8 to +1.5°C; **Figure 1**), easing the reach of foraging threshold (Battisti et al., 2005; Robinet et al., 2007). These beneficial effects facilitating range expansion across Europe have focused attention on late instars larvae, while constraints on other life stages have been overlooked. Yet, the increasing frequency of autumnal heatwaves (Luterbacher et al., 2007) may release the constraint of early frosts on second instar larvae. Warmer summer nights are also expected to accelerate range expansion where female take-off threshold is a limiting factor (Battisti et al., 2006). However, future climate change may also have detrimental effects on the PPM. For instance, the hypothesized prejudicial 25°C threshold is already being reached more frequently in the four areas (+14 to +34 days). Additionally, constraints on winter development of later instars may persist (Robinet et al., 2012) as cold waves may still occur in the 21st century (Kodra and Ganguly, 2014).

Because of cascading phenological effects, the contrasted consequences of climate change on different life stages cannot be considered independent. This may be critical at range margins where populations already face limiting conditions. Range expansion was the slowest in the north-eastern area under continental influence, where warm summers (3a) and early cold snaps (3b) tend to apply antagonistic constraints on PPM lifecycle (3d). Moreover, PPM is widespread across Europe, North Africa and Minor Asia, with locally differentiated phenologies, except in continental regions where opposing constraints can be too strong to be overcome by phenological responses. Conversely, high phenological variability in processions and flights in the south-western area suggests less stringent climatic conditions due to milder summers and winters (**Figure 1C**). Such phenological heterogeneity means that varied life stages experience similar thermal conditions, which in turn alters the survival of colonies in case of extreme climatic events.

Since the early 2000s, an increasing number of erratic PPM phenologies are being reported in France. The most striking illustration is the co-occurrence of both typical (March-May) and atypical (October-January) processions within the northern area under oceanic influence (Boutte, 2012; our own observations in 2006, 2011, and 2014). We expect that the increasing frequency of autumnal heatwaves (Luterbacher et al., 2007) will favor such events of accelerated larval development, but it is unknown whether these phenological discrepancies are viable and maintained over generations. However, in Portugal, a population has durably shifted its phenology toward a summer larval development with higher heat resistance, and co-occurs with typical winter populations (Santos et al., 2011). This illustrates how varied phenologies may broaden the range of climatic conditions the PPM can survive locally, and may ultimately facilitate the persistence or spread to new areas with climate change.

CONCLUSION

Climate warming can exert opposing pressures on the distribution of insects, not only among species, but also within species due to contrasted susceptibility of life stages and heterogeneous phenologies, ultimately altering local survival. Predicting insect distribution under climate change is thus challenging as models should consider not only common concepts of species spread, but also stage-specific thermal thresholds and inter-populational heterogeneity in phenology, acclimation and physiological adaptation (Chuine, 2010; Briscoe et al., 2012; Godefroid et al., 2015). Monitoring both distributional and phenological changes is now crucial to develop and feed such models.

AUTHOR CONTRIBUTIONS

CR and JR prepared the phenology and climate datasets; CR, ML, JR worked on the data and wrote the paper.

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METHODS
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Individual-Based Modeling Approach to Assessment of the Impacts of Landscape Complexity and Climate on Dispersion, Detectability and Fate of Incipient Medfly Populations

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The objective of the presented study was to demonstrate the potential of a bottom-up "ethological" approach and individual-based model of Markov-like stochastic processes, employed to gain insights into the factors driving behavior and fate of the invasive propagule, which determine the initial stages of pest invasion and "cryptic" existence of the localized, ultra-low density incipient pest populations. The applied model, PESTonFARM, is driven by the parameters derived directly from the behavior and biology of the target insect species, and spatiotemporal traits of the local terrain and climate. The model projections are actively generated by behavior of the primary causative actors of the invasion processes—individual "virtual" insects—members of the initial propagules or incipient populations. Algorithms of the model were adjusted to reflect behavior and ecology of the Mediterranean fruit fly, *Ceratitis capitata,* used as a case-example in the presented study. The model was parametrized based on compiled published experimental information about *C. capitata* behavior and development, and validated using published data from dispersion and trapping studies. The model reliably simulated behavior, development and dispersion of individual members of an invasive cohort, and allowed to quantify pest establishment and detection chances in landscapes of varying spatiotemporal complexity, host availability and climates. The results support the common view that, under optimal conditions (farmland with continuous fruit availability and suitable climate), even a single propagule of medium size (100 females) usually results in pest establishment and detection within the first year post-invasion. The results demonstrate, however, that under specific sub-optimal conditions determined by the local climate, weather fluctuations and landscape topography (e.g., sub-urban), the incipient cryptic populations may occasionally continue for several generations, and remain undetected by typical pest surveillance grids for the periods extending beyond 2-years post-invasion.

Keywords: incipient populations, invasive propagule, trapping, pest detection, *Ceratitis capitata*, agent-based model

INTRODUCTION

Some of the most elusive biological phenomena—cryptic existence and fate of alien insect propagules or survival and resurgence of residual populations struggling at the verge of extinction—are actively generated by and critically depend on individual behavior of just a small number of insects, operating within environmental mosaics of the locally fluctuating opportunities, resources and threats. Such incipient populations are inherently vulnerable to stochastic uncertainties (Potapov and Rajakaruna, 2013; Rajakaruna et al., 2013) and essentially, are intractable experimentally. In fairly uniform environments, generic population models, mostly based on "random diffusion" paradigms (Rudd and Gandour, 1985; Kot et al., 2004; Liebhold and Tobin, 2008; Roques et al., 2008), largely explain the growth and spread of the initial populations, and estimate the relation between propagule pressure and probability of establishment (Memmott et al., 2005; Colautti et al., 2006; Drake and Jerde, 2009). But at the ultra-low densities and fine spatial scales of the species-typical daily exploration ranges, translocations of the individual insects are determined by the proximate configurations of environmental attributes and transient availability of resources, and thus are far from random (Lux, 2014; Manoukis et al., 2014; Lux et al., 2016, 2017). It is broadly recognized that during the initial cryptic "latency phase," such small cohorts may linger for some time undetected (Sakai et al., 2001). But in the case of pests of economic concern, duration of the cryptic pest presence and its ultimate success—establishment or resurgence—have immense socioeconomic and regulatory ramifications (Carey, 1996; Papadopoulos et al., 2013; Mcinnis et al., 2017). Consequently, the quest for approaches and methods which could offer new insights into the mechanisms of such ephemeral processes, reveal their key behavioral and environmental drivers and quantify pest establishment and detection chances–is of the utmost practical relevance.

Confronted with the paucity of experimental options, we propose the individual-focused "ethological" approach (Lux, 2014)-stochastic simulation of lifetime events and behaviors of "virtual" individual members of the incipient cohorts, operating under hypothetical agro-ecological scenarios of varying complexity and climates. Such *in silico* emulation of the pest-landscape system offers unique possibility to capture the wealth of information about behavioral particularities of the target insect species, and reflect the impacts of the local conditions with their spatiotemporal dynamics at insect-relevant scales (An et al., 2009; Lux et al., 2016). Importantly, for very small populations scattered at ultra-low densities, such an approach permits more realistic reflection of the individual stochastic uncertainty and non-random behavioral mechanisms of the choices made in locally heterogeneous environment. Once parametrised and validated, the model permits "virtual" emulation of an unlimited number of scenarios—quantification of the net effects of even minor modifications to the topography and/or climate of the studied system (Lux et al., 2016). Such agent-based models, often in combination with cellular automata, can serve as "virtual environmental laboratories" for emulation of complex systems, and are currently used to study ecological and evolutionary processes (DeAngelis and Mooij, 2005; Jovani and Grimm, 2008; DeAngelis and Grimm, 2014), for development of environmental decision support tools (Parker et al., 2002; Parker, 2005; Grimm et al., 2014; Reed et al., 2016), modeling effects of land use and climate change (Louca et al., 2015; Hyandye and Martz, 2017), or site-specific integrated pest management (IPM) (Lux et al., 2016).

The objective of the study presented here was to demonstrate the potential of such an approach for quantification of the latency phase of "cryptic" pest existence, its detectability and establishment chances, in relation to the species-specific pest traits, the initial propagule size, the local landscape topography and climate. For this purpose, an individual-based Markov-like stochastic process model (PESTonFARM, Lux, 2014; Lux et al., 2016) was used, with its algorithms and parameters adjusted to reflect the biology and behavior of *Ceratits capitata*, a well-researched species of enormous economic importance, used here as a case-example. The potential of the presented approach and the model for site-specific assessments of the local pest establishment risks and detection chances, or for optimisation of the pest detection schemes according to the local site topography-was also discussed.

METHODS

Outline of the Model

PESTonFARM (Lux, 2014; Lux et al., 2016) is a site-focused individual-based model, which simulates behavior of individual insects operating within the locally heterogeneous environment. The model consists of two main modules, representing properties of individual "virtual" insects and traits of "virtual" local terrain.

Algorithms of the "virtual insect" module encapsulate relevant information about ecology and behavior of the target insect species, and accordingly, determine (in a stochastic sense) behavior and fate of "virtual" members of the cohorts, which represent the local pest population. Insects constitute suitable objects for such simulation, because their behavior, although intricate, can be reliably described by a set of rules linked to the traits of proximate environs. Individual behavior can be approximated by a Markov-like stochastic process, where response or fate of each insect is stochastically determined only by its current internal state and surrounding conditions (Lux, 2014). Accordingly, each behavioral step, event or "decision" of each individual "virtual" insect is fully randomized and stochastically dependent on its age, reproductive state, and on the local weather conditions, current status of the sector of its actual residence, and where relevant-also that of the nearby sectors.

Local terrain is represented by a set of matrices made by square sectors, with their values representing insect-relevant traits. The trait-values of each sector fluctuate daily according to seasonal changes in host plant phenology, host availability and infestation, local insect density and IPM treatments. Spatial resolution (sector size) is determined by pest biology and its estimated daily mobility ranges (Lux, 2014).

The model uses two key "external" forcing factors: temperature and time. The temperature represents daily average for each season day, while the time factor consists of

successive season days. All phenomena are simulated with 1-day temporal resolution.

Model Adaptation to the Target Species: Mediterranean Fruit Fly, *C. capitata*

The generic PESTonFARM model was developed to simulate behavior and development of frugivorous tephritid fruit flies, which share substantial similarities in their overall biology patterns. Its version 3.1 was adapted and on-farm validated for the European cherry fruit fly, *Rhagoletis cerasi,* and IPM in cherry orchards (Lux et al., 2016). For the study presented here, the model (version 4.1) was adapted and parameterized to reflect the relevant aspects of the biology and behavior of *C. capitata* (medfly). Unlike *R. cerasi,* medfly is a multivoltine species, hence accordingly, a provision was added to simulate overlapping generations and multiple 1-day-spaced discrete age-cohorts. Furthermore, the two species differ quantitatively. Thus, although most of the assumptions and processes described for the version 3.1 (*R. cerasi* model) were retained without major changes, the model was re-parameterized, based on the published information about *C. capitata* and the author's own on-farm observations. Overview of the general assumptions and simulated processes (sub-models) was provided by Lux et al. (2016), while the processes simulated in the *C. capitata* model, adopted estimates of the key parameters and their sources, are presented in **Table 1**.

Model Evaluation

The model was validated by confronting model-generated data with the results of published experiments, used as a point of reference. In all simulated hypothetical scenarios, the assumed conditions broadly resembled that in the reference studies.

General correctness of the simulation of medfly development was verified by confronting the annual population patterns generated by the model for a typical farmland landscape with the results of field experiments from Greece (Papadopoulos et al., 2001). Simulation of the patterns of fly dispersal and trapping was compared with the results of mark recapture experiments conducted by Meats and Smallridge (2007) and Plant and Cunningham (1991). For all fly dispersion scenarios, a homogenous 1 sqkm "virtual" site was generated, containing 1,600 sectors (40 × 40), 625 m^2 each (25 × 25 m). It was assumed that the whole area is covered by uniform pattern of fruit trees, planted in a symmetric grid, with uniform canopies of moderate size (ca. 4 m diameter). Furthermore, eight traps were distributed in pairs, set at the following distances from the site center: 75, 150, 300, and 600 m (**Figure 2**). The traps were assumed to be baited with a lure resembling standard PTA (putrescine, trimethylamine, ammonium acetate) attractant (Ekesi et al., 2007). Simulations were conducted for three different temperature regimes, constant throughout the whole 25-week period: the optimal (25°C) and two other varied by ± 5°C (20° and 30°C). To approximate field conditions, constant 2% daily extrinsic mortality risk due to on-site resident natural enemies and pathogens was assumed, based on the author's own data. To illustrate the modulating effects of the presence of the natural enemies (extrinsic mortalities) on medfly distribution patterns, for the optimal scenario (25°C), an additional case was emulated with no extrinsic mortalities assumed. For each scenario, lifetime behavior, dispersal and trapping of a "virtual" cohort of 1,000 females were simulated. The whole cohort was "released" on day-1 from the center of the site. The virtual flies were allowed to leave the site temporarily, but no immigration was assumed to compensate for the individuals who did not return to the site during the same explorative event. For the most representative simulation from each series, weekly distribution patterns were superimposed and combined into a diagram representing density-pattern of fly presence during the cohort's lifetime. In the "reference" mark recapture experiments (Plant and Cunningham, 1991; Meats and Smallridge, 2007), only sterile insects were used, thus effectively, lifetime dispersal patterns of a single, non-reproducing adult generation were studied. Accordingly, to emulate such experiments, no reproduction was "allowed" for the released "virtual" cohort, i.e., the "oviposition" module of the model was temporarily switched off.

The results were compared with the experimental data reported in the relevant publications. The capacity of the model to reproduce the published experiments and approximate their results was treated as the evidence of correct model calibration. Afterwards, the model was "locked" and used without any further adjustments to its internal parameters.

Virtual Experiments—Simulation of Propagule Behavior, Fate and Detection in Landscapes of Varying Complexity

Virtual Landscapes

Five "virtual" landscapes were generated by the model according to pre-defined parameters (**Table 2**), varied in the degree of their spatial heterogeneity (**Figure 1**). The landscapes, representing 1 sq km of terrain (further referred to as "modeled site" or "site"), comprised 1,600 square sectors, each representing 625 m^2 (25 × 25 m) of land, arranged in 40 × 40 grid. The five types of "artificial" landscapes were designed to approximate typical scenarios, such as fruit production region (further referred to as "Farm Site") with regular 4-hectare blocks of host and non-host trees of regular size, and four variants of peri-urban/urban landscapes (further referred to as "Urban Site 1-4") with different density of buildings, host and non-host trees of randomly varied canopy size, land coverage and host/non-host type. The four landscapes varied in spatiotemporal complexity, fraction of land containing any trees (host or non-host) and the overall host availability, but not in the relative contribution of the host species (always 1:1:1:1) and their respective traits, such as suitability for pest development, phenology, canopy structure and quality. In the landscapes, every grid sector was individually characterized by a range of independent traits, such as: canopy coverage and size, dominant host and its traits, and daily updated about current phenology status, local pest presence, fruit infestation, trap effectiveness, IPM.

TABLE 1 | The main aspects of biology, key processes taken into account, sub-models and adopted parameters.

Aspect	Process/parameter	Adopted values	Relation/sub-model	Basis/source
Adult females	Sex ratio of adults emerging from the soil	1:1	Constant	Assumed
	Pattern of adult emergence from the soil	Staggered, lasting 25–40 days, 75% emerging during the first 10 days	Asymmetric bell-shaped function adjusted to fit the assumed distribution	Assumed, based on own on-farm observations
	Lifespan under constant optimal conditions (20–25°C) and in absence of extrinsic mortality causes	Aver = 79.1, max = 170 days	Gompertz function, calculated according to daily-cohort age, dynamically adjusted to account for seasonally changing temperatures	Based on: Vargas et al., 1997, 2000; Duyck and Quilici, 2002; Manrakhan and Lux, 2006; Grout and Stoltz, 2007; Carey et al., 2008; Nyamukondiwa and Terblanche, 2009
	Intrinsic mortality modeled with 1-day resolution according to individual age	1–170 days		
	Age/maturity categories, at optimal conditions (20–25°C)	1–10 Young, 11–45 Mature, 46–75 Old, 76 + Very Old		
	Extrinsic daily mortality risk caused by the complex of on-farm resident predators and natural enemies	2%	Constant	Broadly estimated, based on own and historic data
Immature stages	Sex ratio and egg status	1:1, 100% fertilized	Constant	Assumed
	Temperature-dependant duration of in-fruit development (from egg to adult emerging from the soil)	Eggs: 3–11 days, Larvae: 8–55 days, Pupae: 9–67 days	Custom-build functions, dynamically adjusted to account for seasonally changing temperatures	Based on: Duyck and Quilici, 2002; Ricalde et al., 2012
	Temperature-dependant stage survival ranges	Eggs: 60–90%, Larvae: 19–80%, Pupae: 26–70%		Based on: Ricalde et al., 2012
	Combined in-soil mortality due to extrinsic factors, e.g., predators etc.	30%	Constant	Broadly estimated, based on own and historic data
Fecundity	Mating status of mature females	Mated (100%)	Constant	Assumed
	Maximum and peak lifetime fecundity (under optimum conditions, temp. 20–25°C)	742 eggs/female, peak at 20–35 days post emergence	Custom-build function adjusted to fit the published data, dynamically adjusted to account for temperature-dependant female maturation pace	Based on: Shoukry and Hafez, 1979; Manrakhan and Lux, 2006
	Intrinsic age-dependent daily fecundity	Range: 0–16, daily individual values generated according to age, assuming normal distribution		
Mobility	Average area covered during a single local exploration errand, used to set the sector size and spatial resolution of all site-related traits	625, equivalent to 25 × 25 m sector	Constant	Assumed based on: preliminary on-farm observations
	Dispersion range	200–700 m	Not programmed, emulated by behavior of 'virtual individuals'	Own observations, and: Meats et al., 2003
	IN/OUT balance between emigration from the modeled site/area and immigration from the neighborhood	1:0.25	Constant	Assumed for all scenarios presented in the paper
	On-site exploration, mobility, and micro-migration	Range and patterns dynamically adjusted according to female maturity, current temperature and local conditions, potential daily averages and SDs calculated according to cohort age, individual values generated based on average and SD	Custom-build age-dependent functions and algorithms adjusted to fit the published data, dynamically adjusted to account for temperature-dependant female maturation pace	Based on own on-farm observations, and: Plant and Cunningham, 1991; Meats et al., 2006; Meats and Smallridge, 2007; Navarro-Llopis et al., 2014; Pimentel et al., 2017
Host phenology, fruit suitability and infestation	Host phenology and fruit suitability for oviposition	Beginning of fruit maturation and suitability, harvest	Species/cultivar-specific	Based on: Papadopoulos et al., 2001
	Host suitability for immature development	Varied, host species specific, ranging from 60 to 100%	Constants	Assumed, partially based on own data
	Daily fruit attractiveness and suitability for larval development	Ranging from 0 to species-specific maximum	Asymmetric bell-shaped function	Assumed, function adjusted to fit data of Papadopoulos et al., 2001
	Harvest accuracy	Varied for hosts, from 40 to 80%	Constants	Assumed
	Local (sectoral) population density	Actual value for each sector		

(Continued)

TABLE 1 | Continued

Aspect	Process/parameter	Adopted values	Relation/sub-model	Basis/source
Detection with baited traps	Trap type & density & bait type	Mc Phail trap (4/sqkm) baited with food-type (PTA) lure	Constant for all modeled scenarios and repetitions (simulation runs)	Assumed, and estimated based on experience and data of: Delrio and Zümreogǔlu, 1983; Lance and Gates, 1994; Kendra et al., 2010; Manoukis and Hoffman, 2014; Meats, 2014; Manoukis et al., 2015
	Trap location	Close the center of site quarter, in a host-containing sector		
	Frequency of bait change	Every 60 days	Constant	
	Daily decline in bait efficacy	0.5%daily	Constant	
	Average daily trapping risk for a newly baited trap, within the sector	5%	Constant within the sector of trap location	
	Effective bait attractiveness area, surrounding baited trap	625 sqm (25 × 25 m)	Constant, uniform within the sector of trap location	
	Responsiveness of females to baited trap	Age dependent, ranging from the initial 40 to 100% at peak, and declining to 50% when 6–7 weeks old and further down to 15% afterwards	Custom build function adjusted to fit the assumed thresholds, dynamically adjusted to account for temperature-dependant female maturation pace	Based on: Manrakhan and Lux, 2006, 2008, 2009; Meats and Edgerton, 2008; Gilchrist and Meats, 2011
Niche utilization	Fruit infestation [%]	Actual value for each sector	Custom build functions, according to type of behavior, with minor impact at low to moderate infestation level	Estimated
	Local (sectoral) daily population density	Actual value for each sector		
IPM	No IPM or any other population suppression actions was assumed	none	Relevant model functions were not activated	Assumed
External forcing factors	Time, season days	1-day resolution	constant	Assumed
	Climate (temperature)	Average daily temperature	Custom-build function, generating annual temperature patterns	Base climate approximating Crete, Greece
	Extreme weather conditions	Calm, no extreme temp. fluctuations, lack of strong winds, rain or hail storms		Assumed

TABLE 2 | Landscape structure parameters.

Site-specific parameters	Farm site	Urban site 1	Urban site 2	Urban site 3	Urban site 4
No canopy (buildings, roads)	0 (%)	20 (%)	40 (%)	60	80 (%)
Host trees	80 (%)	40 (%)	30 (%)	20 (%)	10 (%)
Non-host trees	20 (%)	40 (%)	30 (%)	20 (%)	10 (%)

Canopy parameters (the same for or all sites)	Canopy coverage [%]		Canopy diameter [m]	
	Average	SD	Average	SD
Host trees	50	15	4	1.5
NON-host trees	60	18	5	1.8

Host Trees

The presence of the same four host types was assumed for each modeled scenario, jointly providing suitable fruit throughout the season. The assumption of nearly continuous fruit availability was based on experience from sub- and tropical horticulture, further supported by findings of Papadopoulos et al. (2001). The hosts varied in their phenology, overall attractiveness and suitability for development of the immature pest stages–eggs and larvae. Furthermore, for different hosts, varied harvest accuracy (the percentage of the fruit removed) was assumed, ranging from 60 to 80% (**Table 3**).

Pest Surveillance/Detection

Each "virtual" landscape variant was "equipped" with the same "pest detection scheme." Four pest detection traps, spaced by ca 500 m, were randomly distributed close to the center of each quarter of the modeled area (**Figure 1**). To emulate the usual practice in choosing the exact trap location, suitability of the potential sector was taken into account in order to facilitate the pest presence and detection, thus the trap was located in the nearest host-containing sector. The effective range of bait attractiveness was assumed to approximate sector size (625^2 m).

Propagule Size, Invasion Pattern and Timing

The same invasion scenario was simulated in all cases: a single propagule with 100 females, mimicking a small number of medfly-infested fruits containing 200 larvae (male/female = 1/1) abandoned close to the center of the modeled site. To avoid undue restriction in the propagule establishment chances, the invasion was assumed to start on the 90th day of year (and of March), when the climatic and fruiting conditions were approaching its optimum.

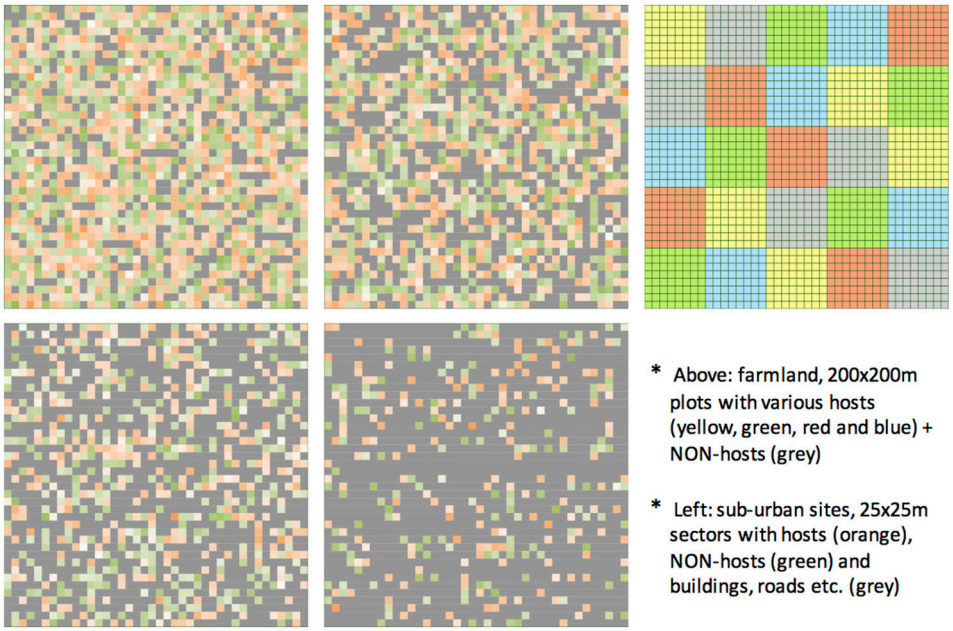

FIGURE 1 | "Virtual" model-generated landscapes of varying degree of fragmentation and complexity.

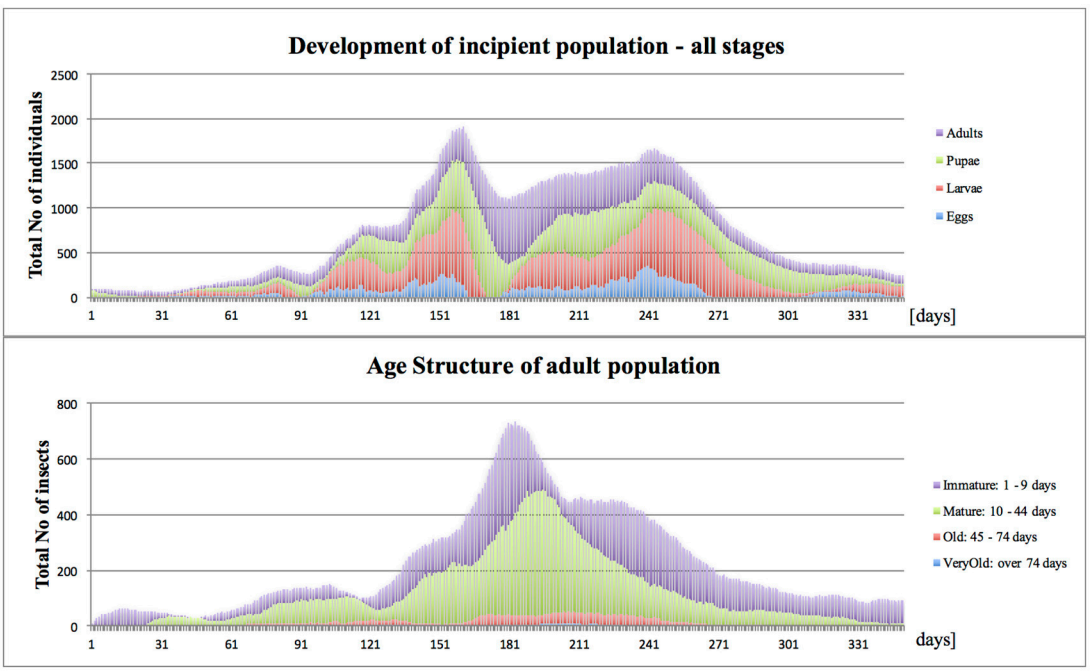

FIGURE 2 | On-farm propagule development in Mediterranean climate (Start, end of March; site, Farm Site; Med climate, average = 20°C, min = 11°C, max = 27°C).

Climate

Unless indicated otherwise, all simulations were conducted assuming mild Mediterranean-type climate (further referred to as "Med climate") with the pattern approximating that of Crete, Greece (the annual min = 11°C, max = 27°C, average = 20°C). Furthermore, for selected cases, simulations were made assuming constant climate (further referred to as "Const") with assumed constant temperature throughout the year at three levels; 20°, 25°, and 30°C, or the optimal climate (further referred to as "Opt") with the annual temperatures following the same annual pattern as the "Med" climate, but fluctuating within more narrow,

TABLE 3 | Host and non-host parameters.

Parameter	Host 1	Host 2	Host 3	Host 4	NON-host
Relative incidence	25 [%]	25 [%]	25 [%]	25 [%]	–
Host suitability	60 [%]	70 [%]	90 [%]	80 [%]	0 [%]
Max attractiveness	70 [%]	90 [%]	100 [%]	65 [%]	29 [%]
Min attractiveness	25 [%]	29 [%]	28 [%]	23 [%]	13 [%]
Fruit availability [days]	80	65	70	85	–
Harvest time [year day]	90	170	250	350	–
Harvest accuracy [%]	65	75	80	70	–

optimum range (the annual min = 20°C, max = 25°C, average = 22.9°C).

Conducive Conditions

To avoid undue bias in emulating the cryptic phase of pest existence, and to clearly expose the effects of the propagule size, terrain structure and climate on the modeled processes, in all emulated scenarios, the following assumptions were adopted, conducive to population development:

- Continuously calm weather throughout the season, without episodes of severe winds, rain or hail storms etc.
- Nearly continuous food and host (fruit) availability within the modeled site.
- Absence of any pest suppression treatments (no IPM, except the four detection traps).
- Lack of significant Allee effect.
- Invasion start at 90th day of the year (end of March), when the temperature and fruit availability is approaching optimum.
- In the "optimal" scenario of Farm Site, the propagule arrives at central plot, which starts fruiting soon after.

Presentation of Results

The model simulates all the processes for each grid sector and season day, and generates a wealth of detailed information. But due to peculiar feature of the incipient cohorts under the study (ultra-small size), the numbers generated daily for each sector, population densities, age structure etc., tend to be extremely erratic and thus of limited practical interest.

Duration of the Simulated Period

The simulation was conducted for 100 weeks (ca. 2 years), except the cases when the population of females present on site (all stages) reached 3,000 individuals, which was treated as a symptom of pest establishment.

Replications

For each landscape and invasion scenario, 15 simulations were executed, to assess development and detectability of the incipient populations founded by the invasive propagules. Each simulation was replicated with the same initialization settings. Auxiliary simulations of the selected cases were replicated 5 times.

Statistical Analysis

The results were presented as averages and either SD or 95% confidence limits. In addition, for comparison of model-generated trapping results with the published trends, a simplified process control test was used, assuming that the process is acceptably controlled (simulated), if the respective experimentally established trend points fall within 3-sigma control limits of the simulated results.

RESULTS

Model Validation

The "Mediterranean" climate (annual min = 11°C, max = 27°C, average = 20°C) broadly resembles the annual pattern of average temperatures in Crete, Greece. With brief seasons of mildly suboptimal conditions and lack of survival-threatening extremes, it is generally conducive for medlfy development throughout most of the year. Summer temperatures (reaching 27°C) only slightly reduced fly activity, and winters (10–12°C) periodically retarded or prevented fly development, and temporarily restricted or stopped their activities. During such periods, the flies remained vulnerable to mortality risks, which varied according to fly developmental stage; eggs, larvae, pupae and adults. The risks included intrinsic temperature- and age-dependent mortalities, and extrinsic ones caused by locally resident predators and pathogens. In optimal conditions (farm site with continuous host availability and lack of excessive spatial fragmentation), an invasive propagule of 100 females released in March, usually was able to establish, and within several months, substantially increase population size and reach detectable levels. The numbers of immature stages reached peak in August and to a lesser extent, also in October-November, while adults–in September (**Figure 2**), which resembles seasonal patterns typical for Greece (Papadopoulos et al., 2001). Also the simulated age structure of adult insects was comparable to that reported from the field (Carey et al., 2008).

Lifetime patterns of fly survival, dispersal and trapping were simulated in a "virtual" homogenous orchard containing fruiting trees, and three constant temperature regimes (20°, 25°, and 30°C). A cohort of 1,000 females was synchronously released from a central point, which mimics a situation of an invasion originating from a single incident of abandoned infested fruit. Female maturation time, the average and maximum lifespan were inversely correlated with temperature (**Table 4**). Accordingly, the lifetime patterns of female dispersal varied with temperature as well (**Figure 3**). In all cases, the females distributed over the whole 1 sqkm area, with a fraction (ca. 5–13%) exploring, at least temporarily, beyond the modeled site (**Table 4**). The area of 90% fly "lifetime" presence was highly clustered around the release point. Its size was temperature-dependent, covering ca. 33–50 hectares (radius from 356 to 444 m) (**Figure 3**), and the respective 80% fly presence zones were smaller, with radius of ca. 300 m, which is in line with the experimental findings of Meats and Smallridge (2007) and Plant and Cunningham (1991).

Fly catches varied strongly with trap distance from the release point, ranging from zero to 11.2 females (0–1.1% of the released cohort). In case of the traps located 75 and 150 m from the release

point, the released flies were detected in each simulation. But the detection became erratic when the trap distance increased to 300–600 m (**Table 4**). Similar to the dispersal patterns, also trap catches and detection chances were positively correlated with temperature (**Table 4**). Simulated relationship between the trap distance and the fraction of the released flies trapped (**Figure 4**) closely fits the trends established by Meats and Smallridge (2007). The 3-sigma control limits of the simulated data points covered the respective trend points, confirming the overall correctness of the model settings and reliability of the generated data. When no extrinsic mortality was assumed (a case of laboratory conditions or a field under intense pesticide and fungicide cover), the average lifespan increased (from 26.7 to 41.8 days). In such conditions, also the 90% dispersal range increased from 45.0 to 56.3 hectares (90% radius from 418.8 to 468.8 m).

TABLE 4 | Effect of temperature on medfly dispersal, trapping and longevity*.

Parameter		20°C	25°C	30°C
90% Fly presence area [hectares]		32.7 (4.94)	45.0 (3.67)	50.5 (3.26)
90% Fly presence radius [m]		356.3 (27.95)	418.8 (17.09)	443.8 (14.00)
Exploration beyond the site [No of females]		44.8 (8.11)	124.2 (4.71)	128.0 (12.06)
Maturation time [days]		16	9	6
Aver. Longevity [days]		35.1 (1.61)	26.7 (0.48)	16.9 (0.44)
Max lifetime [days]		156.6 (12.54)	113.2 (13.74)	79.8 (6.98)
Distance	**Trapping (in 2 traps) and detection**			
75 m	No of trapped females	4.4 (1.14)	7.6 (2.70)	11.2 (2. 17)
	Detections/simulations*	5/5	5/5	5/5
150 m	No of trapped females	1.8 (0.84)	3.0 (1.00)	5.2 (3. 19)
	Detections/simulations*	5/5	5/5	5/5
300 m	No of trapped females	0.4 (0.89)	0.6 (0.55)	1.0 (1.00)
	Detections/simulations*	1/5	3/5	3/5
600 m	No of trapped females	0.0 (0.00)	0.4 (0.55)	0.6 (0.55)
	Detections/simulations*	0/5	2/5	3/5

The table contains averages of 5 simulations/replicates, and SD (provided in parentheses).

Virtual Experiments–Simulation of Propagule Behavior, Fate and Detection in Landscapes of Varying Complexity

All simulated scenarios emulated medfly invasions initiated by a single propagule of 100 females, emerging from a small number of infested fruits "abandoned" at the center of each virtual landscape.

Under "Constant" climates (20°, 25°, and 30°C), with lack of any seasonal temperature fluctuation, the development of the invasive propagule was dependent on both the temperature and the degree of site fragmentation. Durations of medfly developmental stages and successive generations changed according to the temperature, and largely reflected the published data (Ricalde et al., 2012). In spite of the constant temperatures and continuous fruit availability, the host succession forcing seasonal pest shifts within the site, and variation in host traits (attractiveness, suitability and harvest accuracy) (**Tables 2**, **3**), when combined, constituted a mild environmental factor, which jointly with different temperatures and varying site spatial complexity, substantially diversified the trajectories of propagule development and fate (**Figures 5**–**7**).

As expected, and regardless of temperature regime, the largest population growth occurred on the Farm Site comprising large blocks of adjacent diverse hosts. Maximum size of the incipient populations, reached during the first 12 months post-invasion, varied greatly between temperature regimes, and was the highest at 25°C, followed by 30° and 20°C, with ca. 2,400, 1,100, and 300 insects per site (all stages), respectively. Fragmentation of the terrain severely curtailed propagule development, and for all "Urban" sites and temperature regimes, at the peak time, the pest population ranged from ca. 100–270 insects per site. In general, in the "Urban" landscapes, the impact of temperature was less pronounced compared to the farmland, and with the exception of Urban Site 4 at 20°C, the population maxima were broadly similar. However, the population patterns and trends were not consistent, as well as the pest establishment

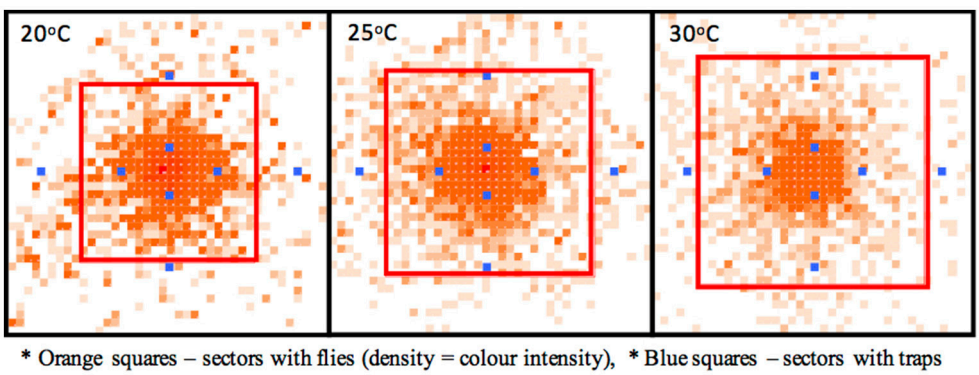

FIGURE 3 | Effects of temperature on dispersion.

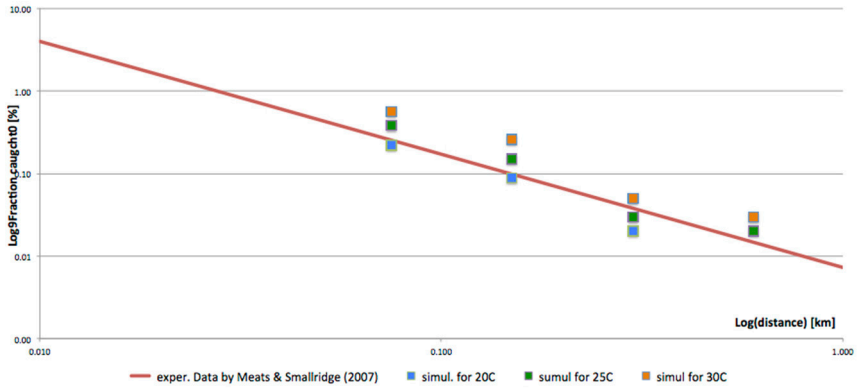

FIGURE 4 | Effects of trap distance on number of females caught.

prospects. In most "Urban" scenarios, after the initial increase, the population gradually declined, and at the end of the first year post-invasion, the numbers of surviving individuals were low, indicating unpromising pest establishment prospects. However, at 25°C, in moderately fragmented sites (Urban 1–3), the incipient populations broadly fluctuated, and at the end of the first year post-invasion, were still at the size suggesting good survival and establishment chances.

It has to be noticed, that the combined effects of temperature and landscape fragmentation were scenario-specific, non-additive and non-linear. Furthermore, these relations shall be expected to heavily rely on the local host configurations; completeness of the annual host succession chain, host traits, spatial arrangement and diversity etc., which compounds the difficulty to generalize the results.

Under the "Optimal" climate (annual min = 20°C, max = 25°C, average = 22.9°C) and optimal site conditions (Farm Site with continuous host availability and lack of excessive spatial fragmentation), the initial propagule quickly followed the generally expected rapid growth trajectory (Mcinnis et al., 2017). Within several months the population established, and reached the densities which, in most cases, assured detection of pest incursion.

Relatively minor modification made to the Mediterranean climate, removal of mild barriers of cool winter and hot summer, substantially facilitated population growth. Compared to the Med case (**Figure 2**), under the optimal conditions (**Figure 8**), during subsequent generations the incipient population reached higher levels, which became the most evident toward the end of the 12-months period, which ended up with ca. 2-fold difference in the number of surviving individuals.

Two years post-invasion, landscape fragmentation, combined with very small propagule size and mild seasonal barriers of the "Mediterranean" climate (annual min = 11°C, max = 27°C, average = 20°C), frequently reduced the incipient populations below viability level. Barring the most extreme scenario (Urban Site 4), the chances for surviving the simulated 2 years period did not differ substantially among the landscapes, and ranged from 60 to 73%. Although generally, in the "urban" landscapes, the survival chances were inversely related to the degree of site fragmentation and host availability. Even when the incipient cohorts survived the two winters, at the end of the 2nd year simulation, the numbers of survivors were frequently below the initial propagule size, with only several individuals left at various stages (eggs, larvae, pupae, adults), which indicated bleak prospects for their future (**Table 5**).

Remarkably, in all landscapes, the incipient cohort usually survived longer that 1 year. The average duration of extinction-ended period ranged from 407 to 567 days (**Table 5**), and usually, was terminated during the second winter. As to be expected, the chances for successful pest establishment were the highest on a "farmland" with solid blocks of host trees (**Table 5**), where after 2 years, the number of on-site residing insects (all stages) increased ca. 10 times, confirming pest establishment. Although even there, it varied, depending whether the initial propagule arrived on a plot containing host trees fruiting soon after the arrival, or on a host bearing fruits later in the season. In the latter case, the establishment chances and the number of insects present on a farm at the end of the 2 year period were lower, and the instances of extinction happened earlier.

Precise estimation of pest detection chances was not our objective; nevertheless, numerous non-detection cases, which occurred within the first 2-years post-invasion (detectability ranging from 66 to 93%), reveal that the detection process of small incipient populations is erratic (**Table 5**). On average, the detection happened ca. 4–8 months post-invasion, thus generally, the first few invading generations usually developed undetected. Interestingly, in the "Urban" sites, the relation between the pest detection chances and the degree of landscape fragmentation was not straightforward. In the most fragmented sites (Urban Site 3 and 4), the pest detection, if occurred, seemed to happen earlier than in the farmland. Furthermore, in the moderately fragmented Site 3, the actual chances for pest detection were the highest (**Table 5**). The latter indicates that a degree of landscape fragmentation may lead to concentration of the insects in fewer spots, which might actually facilitate pest detection. In general, however, with such low density incipient populations, the actual detection seems largely stochastic, which finally tends to occur

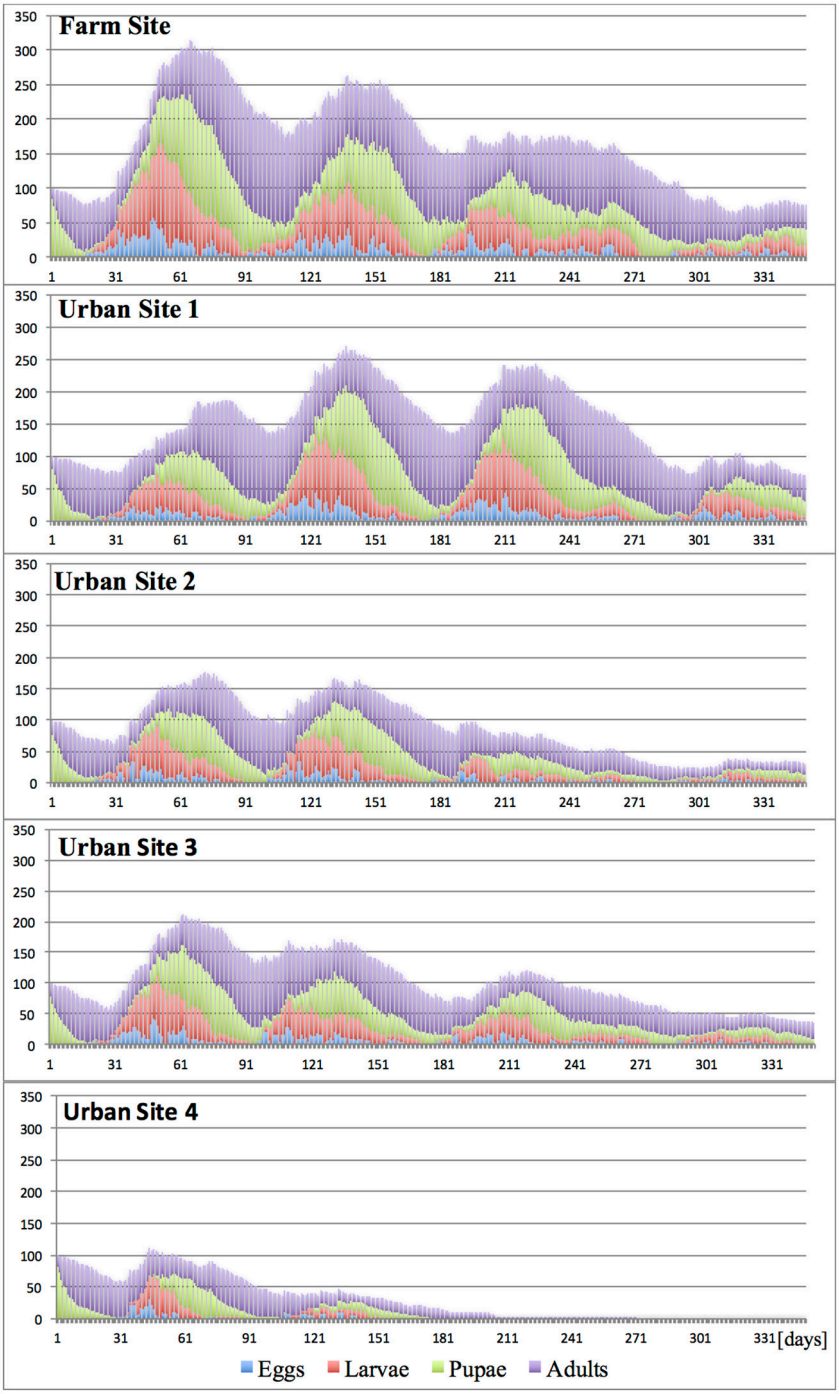

FIGURE 5 | Effects of site fragmentation on propagule development at constant temperature 20°C.

after sufficiently long time. On many occasions, the detection happened well after the incipient population reached its peak, often at the time of its decline, when very low numbers (ca. ca. 40–60) of insects were actually present on farm (**Table 5**). The results demonstrate, that in urban scenarios, quite frequently (ca 35% of cases) such incipient "cryptic" populations may remain undetected for prolonged time, at least as long as 2 years.

DISCUSSION

The objective of the presented study was to demonstrate the potential of individual-based stochastic process emulation model, driven by the parameters derived directly from biology of the target pest species, and spatiotemporal representation of the local terrain and climate. The paper represents bottom-up

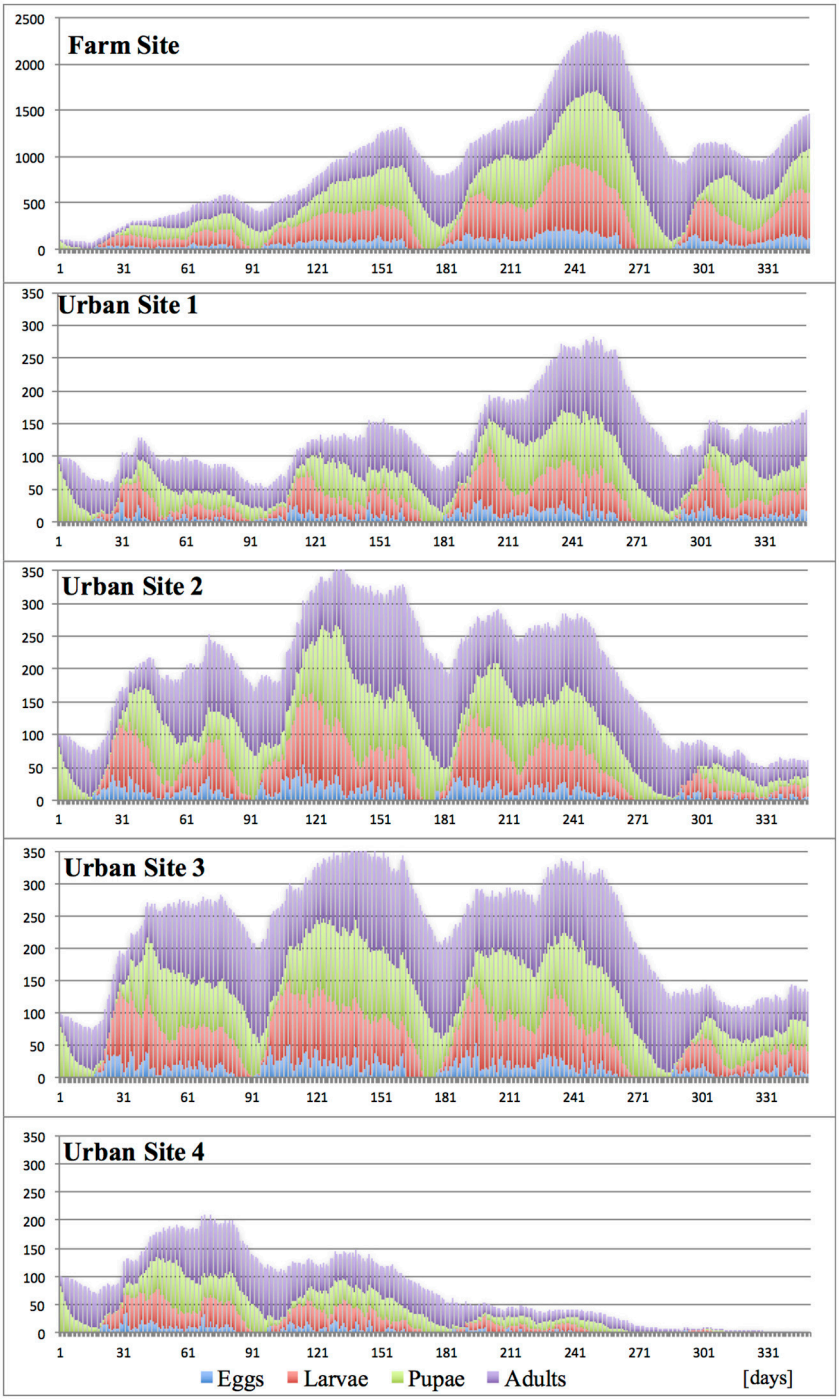

FIGURE 6 | Effects of site fragmentation on propagule development at constant temperature 25°C.

"ethological" approach employed to gain insights into the factors driving the initial stages of pest invasion or "cryptic" existence of incipient pest populations. It is focused on behavior of the primary causative actors of such processes, individual insects-members of the initial propagules or incipient populations. The agent-based modeling approach was chosen because it can adequately reflect the complexity and local specificity of the process, permit concurrent emulation of numerous component sub-processes determining pest behavior and development, and estimate the ultimate process outcomes.

The model, PESTonFARM (Lux, 2014; Lux et al., 2016) was adapted to broadly reflect behavioral and developmental traits of

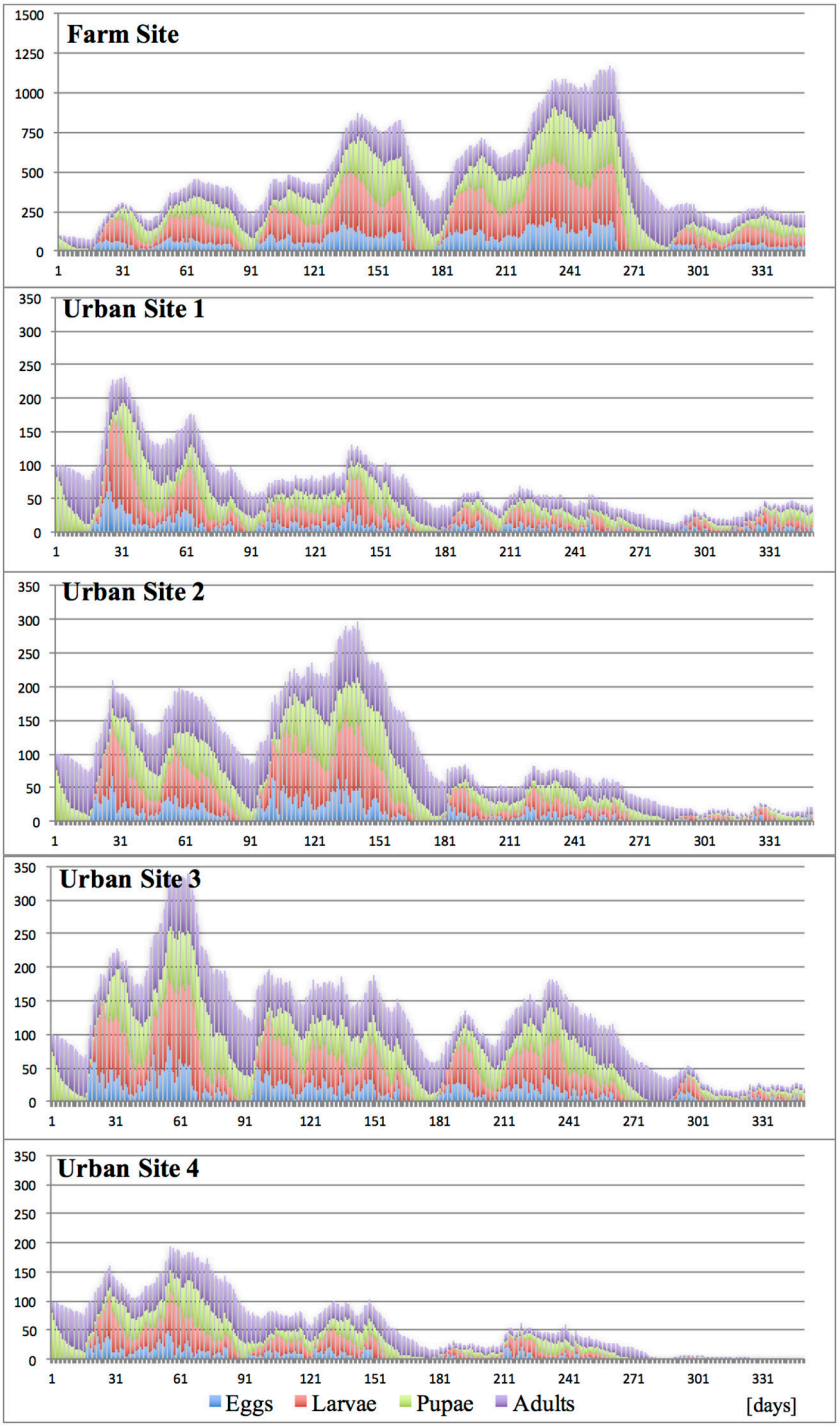

FIGURE 7 | Effects of site fragmentation on propagule development at constant temperature 30°C.

medfly, and used to emulate behavior and fate of the invasive propagules in "virtual" environments, varying in the degree of fragmentation and spatial complexity, host availability, and climatic conditions. The relevance and precision of the model depends on selection of component sub-processes, realism of the assumptions and accuracy of the input data. As may be expected with complex systems, precise quantification of all assumptions, relations and parameters is not feasible. On the other hand, including only rigorously parametrized processes, although tempting to ensure formal methodological correctness, also comes at a price (Lux et al., 2016). Discarding plausible, but only superficially quantified relations *de facto* means adopting

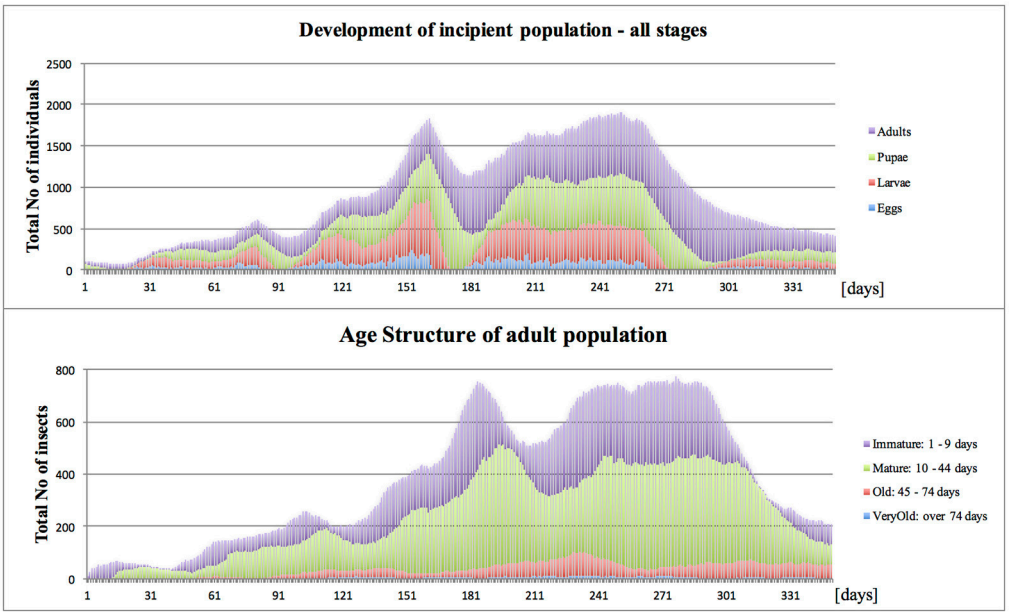

FIGURE 8 | Propagule development in optimal conditions (Start, end of March; site, Farm Site; Opt climate, average = 22.9°C, min = 20°C, max = 25°C).

TABLE 5 | Propagule establishment and detection: Single 100 female cohort, emerging from centrally "abandoned fruit"*.

Parameter*		Farm site	Urban site 1	Urban site 2	Urban site 3	Urban site 4
Medfly survival during 2 years period (any stage) (%)		73.3	66.7	73.3	60.0	6.7
Time, if extinct (day post-invasion)		467.8 ± 86.9	480.0 ± 72.1	567.2 ± 69.4	551.4 ± 88.9	406.7 ± 46.8
Adult survival	Adult survival (%)	53.3	66.7	53.3	46.7	0.0
	No of survivors	164.0 ± 154.2	7.3 ± 2.6	4.5 ± 3.4	5.8 ± 2.9	0.0
	Maximum No during the 2 year period	286.5 ± 160.6	110.9 ± 30.2	144.5 ± 30.4	148.7 ± 31.9	75.3 ± 7.3
Immature survival	Eggs	190.4 ± 171.4	4.3 ± 4.2	0.0	4.5 ± 3.4	0.0
	Larvae	461.3 ± 426.2	15.3 ± 8.8	6.4 ± 4.1	6.6 ± 3.5	6.0
	Pupae	224.0 ± 217.6	5.9 ± 2.3	2.7 ± 1.9	2.0 ± 0.7	3.0
Detection	Detection (%)	86.7	80.0	66.6	93.3	73.3
	Day 1st detected	236.2 ± 88.8	238.3 ± 96.1	204.9 ± 31.1	130.3 ± 30.9	141.2 ± 44.9
	No trapped 1st at detection	1.0	1.0	1.0	1.0	1.0
	No of females at 1st detection	165.9 ± 108.5	57.7 ± 26.7	110.6 ± 37.2	82.6 ± 19.8	47.0 ± 11.9

**Averages and ± 95% confidence limits, based on 15 simulations, each lasting 2 years, "Med" climate, annual min = 11°C, max = 27°C, average = 20°C.*

"hidden," and frequently much less realistic default patterns (zero-order linear relations) for the "discarded" processes. The difficulty in balancing the trade-offs between conceptual simplicity and clarity of the model, process complexity vs. our insight and data availability, model realism, generality and utility, is already recognized (Evans et al., 2014; Evans and Moustakas, 2016; Moustakas, 2017). Simple models built on generic assumptions, sometimes based on superficial analogy borrowed from other disciplines, such as random "particle like" insect dispersion, or continuous "area-wide" probability-field of an insect being caught even in a distant surveillance trap, etc. may provide sufficient approximation of large pest incursions at high population densities. For very small propagules or incipient populations at ultra-low densities, when the process outcome depends on a complex behavior and stochastically uncertain fate of a few individual insects operating in a locally complex environment, such models may still offer generic predictions which might be statistically relevant for a pool of events made of hundreds of individual invasion cases, but are likely to be of little relevance to a particular case or location.

Our model is focused on the causative actors of the early invasion process, and the complexity of their individual behavior.

Hence, a pragmatic approach was taken-including into the model also putative and, in some cases, only tentatively parametrized processes, and verifying the model in confrontation with experimental data. The aim was to construct a "frame-model" for holistic emulation of the pest-landscape system at the insect-relevant scale, which could be fine-tuned once more precise information becomes available. Merits of such approach to the agent-based models were demonstrated in earlier studies (An et al., 2009; Lux et al., 2016). The same pragmatic approach was used in modeling climatic conditions. Although it is possible to use in the model hourly temperatures, the realistic gain in the precision of generated projections is usually problematic, apart from largely false "impression of the precision." This is due to the fact that hourly temperatures are usually available from more or less distant weather stations, where the conditions and terrain are usually different. Even more importantly, in heterogeneous landscapes containing diverse tree patterns with varied canopies, the local temperatures in the spots where the individual flies actually live and operate greatly vary within the whole site, and also within individual tree canopies. Fruit flies, such as *R. cerasi,* are known for their capacity to actively seek suitable spots within the canopy, which may vary diurnally and depend on fly age (Lux et al., 2017). Because our aim was to evaluate generic scenarios, and not to provide precise pest forecasting, using average daily temperatures as the external forcing factor for the simulation process was deemed acceptable, which also provided the added benefit of increased model efficiency.

The results confirm that the model, although still tentative, generates projections closely mirroring the experimental data about temperature-dependent medfly development (Ricalde et al., 2012), age structures of field-established population and the presence of variable fractions of "old" flies, as documented by Carey et al. (2008). Also, the on-site dispersion of invasive propagules and catches by the traps located at various distances to the invasion spot-was emulated in agreement with the trends experimentally established by Meats et al. (2003, 2006), Meats and Smallridge (2007). It has to be recognized, however, that in spite of the fact that medfly is among the most intensely studied pests, our understanding of the behavior and ecology of wild populations living in complex natural conditions still remains limited and incomplete. Most of our knowledge is based on the laboratory or, to lesser extent, semi-field experiments executed in spatially homogenous agro-landscapes, mostly conducted with lab-reared flies, more often than not, originating from the colonies kept for many generations in highly artificial conditions. Although it is known that behavior of wild, locally established flies may differ compared to the newly released lab adapted insects, sometimes directions and magnitudes of such discrepancies may be difficult to estimate or, occasionally, might even contradict the "common sense" expectations. For example, the fraction of "old" individuals in wild populations in Greece was found to be much higher than previously thought, and although it is generally believed that the lab-adapted flies will live longer in lab conditions than the newly collected wild ones, in fact the residual life expectancy of the wild-trapped flies, when, maintained in optimal lab conditions, was found to be longer compared to their lab-adapted counterparts (Carey et al., 2008). Furthermore, medfly behavior, its diurnal and lifetime patterns strongly depend on the individual age, maturity and nutritious status (Manrakhan and Lux, 2006, 2008), or even conditions and scale of the experimental set-ups used during observations (Manrakhan and Lux, 2009). Even the seemingly simple phenomenon of propagule dispersion, is *de facto* a complex process-a combined outcome of several sub-processes, independently moderated by ambient temperature, individual insect age, site spatiotemporal structures etc. They include, for example varied pace of female maturation, variation in survival rates, age- and status-dependent propensities to undertake various activities, lifetime changes in the patterns of movement, etc. These seemingly unrelated processes, with inherent stochasticity components, jointly translate into individually different "intrinsic" lifespans, further curtailed by varied durations of individual exposures to extrinsic mortality factors, such as the local activity of natural enemies and pathogens. Last, but not least, distributions are modulated also by seasonally changing patterns and traits of the terrain and patterns of the local IPM practices, presence or absence of host trees, their spatial arrangement, cultivar composition and canopy structures, fluctuating phenology status etc. The resulting dispersion patterns constitute the overall outcome of all these processes. For these reasons, the models based on the random dispersion paradigm usually provide acceptable approximations for larger populations (e.g., several thousand individuals) in larger and fairly homogeneous environments, but are less adequate to study the initial ultra-low-sized cohorts (from a few to few hundred) operating at the local and highly heterogeneous scales. The presented model emulates the abovementioned sub-processes at the scales relevant to the usual ranges of medfly explorative behavior, and generates dispersion and trapping results which largely remain in line with the experience and the published experimental findings (Duyck and Quilici, 2002; Meats et al., 2003, 2006; Manrakhan and Lux, 2006, 2008, 2009; Meats and Smallridge, 2007; Carey et al., 2008; Ricalde et al., 2012).

To demonstrate the potential of the model to provide preliminary insights into general trends in medfly behavior at the early stages of invasion, fate of a 100-female propagule was emulated in "virtual," model generated landscapes, varying in the degree of their fragmentation and complexity. In each 1 sqkm modeled site, four pest monitoring traps were located, which broadly approximates the usual trap densities employed in intense pest surveillance grids, like the ones routinely used in Australia (Meats, 2014) or California (Manoukis et al., 2014). As reported by Meats and Smallridge (2007), also in our simulations, the detection within the lifespan of the first invading generation occurred very seldom when the propagules arrived at the spot not immediately proximate to the trap. But contrary to the "common wisdom," many times, the detection finally happened not at the time when the incipient population was at its maximum, but much later (4–9 months post-invasion), sometimes during the population decline phase, when the actual numbers of the adult flies present on site were surprisingly low (10–100 individuals/km^2). This indicates, that in the case of ultra-small incipient populations, the detection process is highly random,

related not only to the incipient population size, but largely also to the "persistence" of the trapping exercise.

As commonly expected (Mcinnis et al., 2017), the invasion into a farmland frequently resulted in rapid pest multiplication, establishment and detection. However, even in such "conducive" environment, the pace of population growth and timing of its detection was modified by a "good luck" element-whether the initial propagule arrived at the spot about to bear fruit soon, or at a plot containing a host fruiting much later in the season. In the latter case, the initial need for broad dispersion to reach the plots with suitable host substantially reduced the invading cohort and retarded the population growth. Alike, in the climates with seasonally suboptimal conditions (cool winter or very hot/dry summers), the timing of the invasion largely determines the prospects for propagule development and establishment.

In more fragmented landscapes, broadly representing various sub-urban scenarios, the processes of pest establishment and detection become progressively more erratic along with the increase in site fragmentation and complexity. In many cases, the detection occurred after more than a year post-invasion, and on several occasions failed during the whole 2 year-long modeled period, despite continuous pest presence in the area. The set of "conducive" assumptions adopted in all reported simulations, in particular the assumed lack of significant Alee effect, propagule arrival at the pest-suitable time of the year, continuous host presence and moderate extrinsic in-the-soil mortality (30%), absence of "ephemeral" random incidences of bad weather etc., combined together, likely caused a degree of overestimation of the pest establishment and detection chances. In view of this, the obtained results, demonstrating the possibility of occasional "cryptic" existence of the pest, which can remain undetected by the pest surveillance scheme for prolonged period of a few years, gain in credibility. Schematic relations between the degree of site suitability and uncertainty and likely position and breadth of the "cryptic" pest phase are presented on **Figure 9**. Including into the simulations random mildly "unconducive" conditions mentioned above, in most cases broaden the "extinction zone," but will not eliminate the "cryptic" phase, rather will increase its breadth and shift the "cryptic" zone down the "site suitability and uncertainty" continuum, presented on **Figure 9**. Between the two broadly recognized and largely predictable scenarios of a localized pest invasion (Mcinnis et al., 2017): rapid establishment and detection or propagule extinction (in optimal or adverse conditions, respectively), there is a "continuum of uncertainty" of variable extent, with cryptic pest presence and its erratic detection, conveniently ignored by simplistic approaches. Currently, paucity of experimental data and lack of experimental methods hamper quantitative exploration of this "uncertainty zone." The presented approach and model, once improved, could permit to tackle this phenomenon.

Such a result is not unusual. In conservation biology, the possibility of long cryptic existence of the species considered extinct since many years and surviving undetected in small numbers—is widely recognized and well documented. On many occasions, their continuous existence in the area became revealed only by sudden "re-discovery" or resurgence caused by environmental change. Examples are abundant, and include even seemingly difficult to overlook large mammals, such as Bridled Nailtail Wallaby, "re-discovered" near Dingo, Queensland, Australia, after being thought extinct from 1937 to 1973 year (Australia Wildlife Conservancy)[1], or Gilbert's potoroo, last sighted 1869 and, after a thorough search conducted in the 1970s, thought to be extinct, until its rediscovery in 1994 in well researched Two Peoples Bay Nature Reserve in Australia (New Scientist, 1994)[2]. Similarly, resurgence of several indigenous fish species was observed after invasion of water hyacinth at Lake Victoria in Africa (personal observation) or after overfishing the earlier introduced alien top predator, the Nile perch (Balirwa et al., 2003; Chapman et al., 2003).

Also in fruit flies, in the areas with well-established and broadly spread medfly populations (e.g., in Greece), seasonal interruptions in our capacity to monitor or detect the pest presence, either by fruit sampling or trapping, are well known from the experience and are documented in the literature (Papadopoulos et al., 2001). In fact, such situations are fairly typical for the areas with regular periods of suboptimal climatic conditions, hampering insect development, reproductive and explorative activities, and temporarily reducing their responsiveness to attractants. During such seasons, the pest population is also periodically decimated to very low or residual levels, which, upon the onset of more favorable conditions, especially in farmland landscapes with sufficient continuity of host presence, rapidly builds up again and reaches easily detectable levels.

Very small invasive propagules, founding highly localized incipient populations, present more complex case. Their precarious existence is threatened by even broader range of adverse factors, which can push them into the peril of extinction (non-establishment) or maintain for some time at low levels. The possibility for substantial delays in detecting such point-source invasions even in favorable conditions, extending beyond generation time, was already reported (Meats et al., 2003; Mcinnis et al., 2017). Favorable incidents, such as transient suppression of the local natural enemies, local alteration of soil cover temporarily reducing mortality of the flies emerging from soil, or random availability of "un-harvested" suitable fruit source, permit such incipient populations, lingering at the verge of extinction, to expand and reach detectable and economically concerning levels. On the other hand, seasonally occurring mild suboptimal conditions combined with landscape fragmentation periodically restrict the cohort size, and extend the cryptic (undetected) period of pest presence. The latter could be extended much further, if the already small local cohort becomes randomly attenuated by occasional spells of unfavorable weather, even short breaks in the local annual "fruit-chain," more accurate local harvests or IPM treatments etc. Such randomly occurring events, which nevertheless might constitute typical feature of some locations, will repeatedly inhibit the

[1] http://www.australianwildlife.org/wildlife/bridled-nailtail-wallaby.aspx Accessed March 14, 2017

[2] https://www.newscientist.com/article/mg14419562-300-potoroos-return/ Accessed March 14, 2017

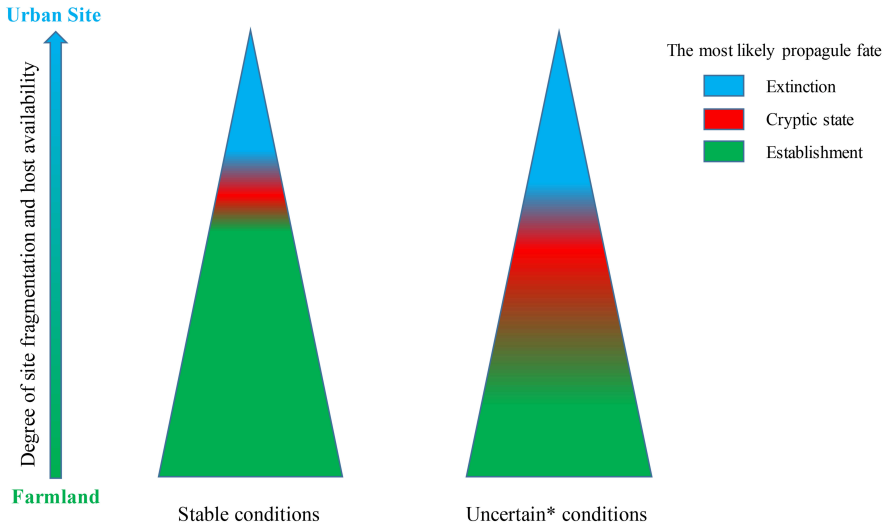

FIGURE 9 | Occurrence of medfly "cryptic" phase under periodically suboptimal conditions of varying uncertainty.

population growths or reduce it back close to the starting levels, maintaining the "fluctuating equilibrium" state of the incipient cohort, thus substantially extending the duration of its "cryptic" phase.

In addition to the fundamental and scientific merits of exploring this elusive biological phenomenon, in the case of medfly, the question of realistically plausible durations of the "cryptic" existence of undetected incipient populations has immense socioeconomic and regulatory ramifications, leading to controversy in estimations of its extent. It is generally accepted that under favorable host and climatic conditions the invading flies will either not survive or will reproduce rapidly, and thus are likely to be detected within the first few generations (Mcinnis et al., 2017; Shelly et al., 2017), which was also demonstrated by our simulations. However, the possibility that such populations could remain undetected for more extended periods (years) is widely questioned (Mcinnis et al., 2017; Shelly et al., 2017). Consequently, recurrent pest detections in the areas deemed pest-free are then attributed to random new invasion incidents, further justified by analysis of trade, commodity and human movements (Szyniszewska, 2013). On the other hand, the documented pest detections in largely the same (or similar) and, frequently, sub-urban environments, recorded at stochastically similar intervals ranging several years, provide grounds for the opinion about the possibility of longer durations of the "cryptic" pest phase (Carey, 1996; Papadopoulos et al., 2013; Carey et al., 2017), stretching beyond the already accepted periods of a few generations (Mcinnis et al., 2017). Because of the inherent experimental "intractability" of the incipient populations existing at the ultra-low densities, both the opinions postulating the occurrence of several-year-long cryptic phase, as well as the views negating such possibility-are largely based on indirect evidence.

In this context, it has to be emphasized that the presented results do not allege to represent any "real" situation in any particular country/location, or discuss regulatory implications of the biological phenomenon of variable durations of the "cryptic" pest phase. The results illustrate hypothetical scenarios and "general" relations between the fate of invasive pest propagule, landscape topography and climate, and demonstrate the capacity of the proposed approach to comprehensively emulate the relevant processes and quantify their outcomes. The presented stochastic individual-based model, based on strictly "insect-focused" approach, has the capacity to offer plausible projections, solely based on experimentally documented knowledge about individual pest behavior and the relevant traits of the local environment. The presented results demonstrate, that such approach offers new, pest-biology-based insights into the details of this elusive process.

To progress beyond this "potential-demonstration" stage, several information gaps about medfly ecology shall be filled, in order to substitute some of the experience-based estimates used in the model with more accurate experiment-based data. As demonstrated earlier in the case of the European cherry fruit fly, *R. cerasi,* the model, when parametrised to reflect "real" local site topographies, IPM conditions and climates, and tuned to emulate specific behavioral traits of the locally-acclimated on-site residing insects, can generate fairly realistic and accurate assessments of diverse site-specific scenarios and provide guidance for the local IPM improvement (Lux et al., 2016). Thus, potentially, the model could serve to identify important gaps in our knowledge about the ecology and behavior of incipient fruit fly populations, estimate impacts of various environmental traits and topography arrangements, and possibly, exploit the local spatiotemporal landscape heterogeneity for enhancement of the local pest surveillance schemes.

CONCLUSIONS

1. The model reliably simulates propagule behavior and development, its fate and detection in landscapes of varying spatiotemporal complexity, host availability and climates.
2. The results support the common view that, under optimal conditions (farmland with continuous fruit availability and suitable climate), even a single propagule of medium size (100 females) usually results in pest establishment and detection within the first year post-invasion.
3. The results demonstrate, however, that under specific sub-optimal conditions determined by the local climate and landscape topography (e.g., sub-urban), the incipient phase may occasionally continue for generations and stretch beyond 2-years post-invasion, being undetected by typical pest surveillance grids.

AUTHOR CONTRIBUTIONS

SL conceived the project, contributed proprietary generic PESTonFARM model, made all model adaptations (conceptual modifications, algorithm & code writing, assumptions & calibrations), executed and interpreted all the simulations used in the manuscript, and wrote the manuscript.

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Ectotherms in Variable Thermal Landscapes: A Physiological Evaluation of the Invasive Potential of Fruit Flies Species

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Climate change and biological invasions pose one of the greatest threats to biodiversity. Most analyses of the potential biological impacts have focused on changes in mean temperature, but changes in thermal variance may also impact native and invasive organisms, although differentially. We assessed the combined effects of the mean and the variance of temperature on the expression of heat shock protein (*hsp90*) in adults of the invasive fruit fly *Drosophila melanogaster* and the native *Drosophila gaucha* in Mediterranean habitats of central Chile. We observed that, under these experimental conditions, *hsp90* mRNA expression was higher in the invasive species but absent in the native one. Apparently, the biogeographic origin and niche conservatisms are playing a role in the heat shock response of these species under different putative scenarios of climate change. We suggest that in order to develop more realistic predictions about the biological impact of climate change and biological invasions, one must consider the interactions between the mean and variance of climatic variables, as well as the evolutionary original conditions of the native and invasive species.

Keywords: global change, environmental variability, physiological acclimation, heat shock proteins

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INTRODUCTION

The question about what makes an exotic organism a successful invader has been central in the fields of applied ecology and environmental protection the last decades (Kolar and Lodge, 2001; Seastedt, 2009). Current research points out to a mix between individual and community or ecosystem features. In the community approach, focus has been on the differential characteristics of susceptible and resistant communities (Hector et al., 2001), like the hypothesis of diversity—invasiveness. At individual level the search of traits that predict the invasive potential of a species spans from reproductive potential and foraging habits to environmental tolerances (Sol et al., 2012; Bates et al., 2013; Capellini et al., 2015). It is in the later where thermal physiology provides the conceptual framework needed to explain the success—failure pattern of exotic species over the thermal landscape.

How it has been previously described (Helmuth et al., 2010; Bozinovic et al., 2011a,b; Estay et al., 2014), insights about the suitability of a thermal landscape for a given species should make reference not only to average values, but also to the intrinsic variability of

perceived temperatures (Bozinovic et al., 2016a,b). This is a key point at predicting future changes due to climate change, where theoretical (Katz et al., 2005) and empirical approaches (Easterling et al., 2000) indicate that global warming impacts not only the mean temperatures, but also the magnitude of dial and seasonal variation in temperature (Vazquez et al., 2016).

Ectotherms are particularly susceptible to temperature variation, as their body temperature is determined to a large extent by environmental conditions (Hoffmann et al., 2003; Karl et al., 2009). The ability to cope with extremes rather than different mean temperatures is probably of much greater importance for species survival and thermal adaptation (Anderson et al., 2003). The most studied physiological mechanism to cope with extreme temperatures is through the expression of stress-inducible heat-shock proteins (HSPs). These, chaperone proteins minimize the problems that arise when other proteins are in a non-native conformation (Feder, 1999; Feder and Hofmann, 1999). Most HSPs participate in protein folding and unfolding, and they are essential in cellular responses to a variety of damaging conditions (Parsell and Lindquist, 1994). Most of our knowledge about shifts in gene expression in response to changes in temperature comes from studies of the heat- and cold-shock responses (Feder and Hofmann, 1999; Johnson et al., 2009; Zhang and Denlinger, 2010). However, these studies typically focus on a narrow range of high or low temperatures that are severe and induce a strong cellular stress response. Very few studies have addressed changes in gene expression associated with routine daily or seasonal temperature regimes experienced by organisms (Podrabsky and Somero, 2004).

A simple hypothesis relating invasiveness and HSPs indicates that HSP expression is high in invasive species (Kelley, 2014); however, a new question arise: which is the base line to compare expression levels?. An elegant solution is compare expression levels between close related non-invasive and invasive species. Some evidence pointed out that invasive species are more eurythermal than natives, i.e., have the ability to maintain physiological function over a wide range of temperatures. Unfortunately, the few studies that compared temperature tolerances between invasive and native non-invasive species have shown conflicting results (see Kelley, 2014 meta-analysis). This lack of agreement in the results could be a consequence of the importance of the evolutionary history on the current status of traits linked to thermal tolerance. In this sense, the biogeographic origin of each species is a key component in this kind of comparative analysis.

Here, we experimentally test the effects of potential scenarios of climate warming given by changes in mean temperature and thermal variance on the heat shock protein response (*hsp90* mRNA) of an invasive and a native non-invasive species in central Chile. Specifically we address the following questions: (1) Does the expression of transcripts encoding for hsp90 vary across mean temperature and thermal variance treatments? (2) Does the expression of transcripts encoding for hsp90 vary between native and invasive species? and (3) How does putative climate change interact with the biogeographic origin of native and invasive species in the expression of hsp90 mRNA?

MATERIALS AND METHODS

Model Species

Our study use two species of *Drosophila* as a model for answering our questions: *D. melanogaster* and *Drosophila gaucha*. The former is an invasive species with a tropical origin whose range expansion may be associated with human activities (Keller, 2007). According to the entomological literature, *D. melanogaster* was absent from Chile until 1888 (Blanchard, 1851; Reed, 1888), and the first records from Chile can be found in the work of Sturtevant (1921). On the other hand, *D. gaucha* is a native species that exhibit a comparatively smaller geographic range with Andean high-altitude origin and still inhabiting in their original range (Budnik and Brncic, 1974; Brncic, 1987). Nevertheless, interestingly, these two species coexist in nature where they exhibit similar life modes, food habits and reproductive sites (Godoy-Herrera and Connolly, 2007). Adult flies were collected in Til-Til (33°05′S, 70°55′W at 586 m above sea level). The climate at this locality is Mediterranean, with an annual mean precipitation of 376 mm, concentrated 65% in winter, from June to August. Precipitation is minimal from December to March, accounting for only 3% of the yearly total. Temperatures are highest from December to March (mean = 22°C), corresponding to austral summer, and lowest from June to August (mean = 7°C), during austral winter. Boher et al. (2010, 2012) describe the range limits of both species and populations at different acclimation temperatures. In the case of *D. melanogaster* upper lethal limit range from 36.7 to 37.8°C, and the lower lethal limit from −5.1 to −3.7°C. On the other hand, *D. gaucha* showed a upper lethal limit ranging from 35.9 to 37°C, and a lower lethal limit ranging from −11.3 to −5.5°C.

Culture and Experimental Design

We used the fourth generation of field collected adults of *D. melanogaster* and *D. gaucha* to avoid potential environmental and maternal effects. Flies were reared in mass at 24°C in 250 ml glass vials with Burdick (1954) culture medium. At each generation, 40 adult flies were collected randomly from the rearing vials and transferred to fresh vials. After 3 days the adults were removed to prevent overlap between generations. Temperature range was set according to Boher et al. (2012) *Drosophila* thermal limits. Based on Bozinovic et al. (2011b) experimental design, during 15 days, adult flies were randomly assigned to four thermal treatments in climatic chambers; 17 ± 0°C (low mean, no variance = 17C), 17 ± 5°C (low mean, high variance = 17V), 24 ± 0°C (high mean, no variance = 24C), and 24 ± 5°C (high mean, high variance = 24V). The photoperiod was L:D = 12:12 h. 24°C was used as control temperature. We used hsp90, a molecular chaperone member of the heat shock protein family, which is upregulated in response not only to heat but also to cold stress (Colinet et al., 2010). After rearing flies at constant or fluctuating temperatures, we quantified *hsp90* mRNA expression in both species in each thermal scenario. Also hsp90 was the protein with more conserved alignment sequences in the primer design step. This is particularly relevant because *D. gaucha,* contrary to *D. melanogaster,* is not a model study so

conserved alignments are critical to ensure an adequate primer performance.

RNA extraction was performed on 16-days old adults, after the 2 week acclimation period using Total RNA miniprep kit (Sigma). Each extraction was originated from a pool of 10 flies with three replicates per treatment. One microgram of total RNA was used in reverse transcription to cDNA, using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Coding sequences of *hsp90* target gene and *rp49* housekeeping gene were retrieved from the GENBANK database. PCR primers were designed using Primer 3 module as follows: *hsp-90* forward 5′-CAAATCCCTGACCAACGACT-3′, *hsp-90* reverse 5′- TGATGTTGTTGCGCTTCTTC-3′; *rp49* forward 5′-CACCGGATTCAAGAAGTTCC-3′, *rp49* reverse 5′-GACGATCTCCTTGCGCTTCT-3′.

Real-time PCR were performed on a LightCycler 480 (Roche) system. PCR reactions were carried out using iQ SYBR Green Super Mix (Bio-Rad), and the crossing point (C_p) were obtained. Samples were subjected to PCR amplification at 95°C for 5 min, 40 cycles at 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s. A dissociation curve was carried out to ensure that there was only one product. A control without template was included in all batches. Amplification efficiency of each gene was validated by constructing a standard curve through four serial dilutions of cDNA. Data were analyzed following a method based in C_p according to Pfaffl (2001).

Statistical Analysis

Statistical analysis of gene expression values was carried out using the REST 2008 program (Relative Expression Software Tool V 2.0.7; Corbett Research; Pfaffl et al., 2002). This program calculates changes in gene expression between two groups, control and sample, using the corresponding distributions of C_p-values as input. The program makes no assumptions about the distributions, evaluating the significance of the derived results by using the Pair-Wise Fixed Reallocation Randomization Test tool (Pfaffl et al., 2002).

RESULTS

All qRT-PCR assays yielded specific products (i.e., single melting peak). The acclimation temperature of 24°C was used as control in *hsp90* expression analysis as was the rearing temperature for both species. The heat shock response varies greatly between the two species. *D. melanogaster* subtly increase *hsp90* mRNA expression when acclimated at 24V (**Figure 1**). The expression of *hsp90* mRNA was upregulated in *D. melanogaster* after acclimation at 17°C and also after acclimation at 17V without significant differences between them (**Figure 1**).

Statistical analysis using the REST 2008 program (**Table 1**) indicates that *hsp90* mRNA expression in the invasive *D. melanogaster* in both, low mean and low mean–low variance treatments, were significantly higher with respect to the other treatments and to the other species (fold change of 3.99; $P = 0.027$ and 3.13; $P = 0.048$, respectively). On the other hand, the native species, *D. gaucha* did not show *hsp90* mRNA overexpression at any of our thermal treatments (**Table 1**; **Figure 1**).

DISCUSSION

Adaptation to varying thermal environments depends on the temporal pattern of environmental changes and the physiological tolerance of each phenotype (Cavieres et al., 2016). In spite of the well-known role of climate change on biodiversity (Burroughs, 2007; Angilletta, 2009; Chown et al., 2010), the range of thermal conditions in time and space, its variability and how invasive and

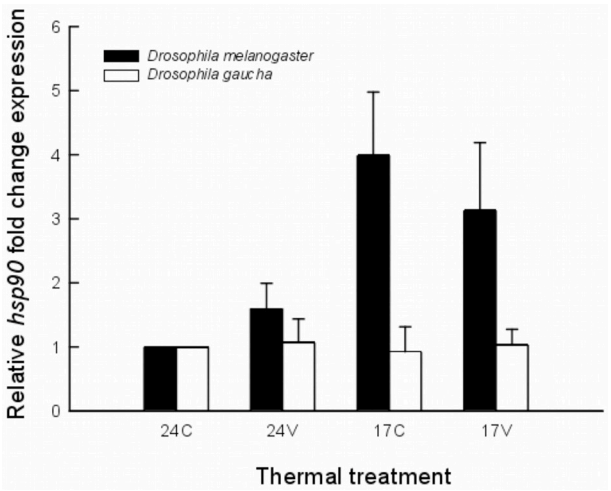

FIGURE 1 | Expression levels of *hsp90* mRNA in an invasive (*Drosophila melanogaster*) and a native (*Drosophila gaucha*) species acclimated to four thermal treatments. Treatments were: 17 ± 0°C (low mean, no variance = 17C), 17 ± 5°C (low mean, high variance = 17V), 24 ± 0°C (high mean, no variance = 24C), and 24 ± 5°C (high mean, high variance = 24V). Results expressed as copies of hsp90 per copies of rp49. Data is representative of three biological replicates.

TABLE 1 | REST statistical analysis data of the expression values of *hsp90* and the range of standard errors (*SE*).

Species	Thermal scenarios	Fold change	SE	*P*-value	Result
D. melanogaster					
	17 vs. 24C	3.991	3.558–4.544	0.027	UP
	17V vs. 24C	3.131	2.612–3.670	0.048	UP
	24V vs. 24C	1.602	1.429–1.819	0.056	–
D. gaucha					
	17 vs. 24C	0.924	0.746–1.136	0.622	–
	17V vs. 24C	1.035	0.928–1.162	0.751	–
	24V vs. 24C	1.067	0.890–1.260	0.465	–

Data is representative of three biological replicates per treatment, each replicate originated from a pool of 10 flies. Data indicate that hsp90 is significantly upregulated (UP) only in individuals of the invasive species acclimated to constant and variable cold environmental conditions. Thermal treatments are: 17 ± 0°C (low mean, no variance = 17C), 17 ± 5°C (low mean, high variance = 17V), 24 ± 0°C (high mean, no variance = 24C), and 24 ± 5°C (high mean, high variance = 24V).

native animals respond to different climate change scenarios are still puzzling.

Bozinovic et al. (2013, 2016a,b) showed that those ectotherms that are continuously exposed to variations in environmental conditions deal with this variability through thermal acclimation and/or acclimatization, which impacts on the survival of natural populations. These authors also propose that if short time thermal variability changes in any of the directions forecast by climatologists, physiological approaches are necessary to predict the biodiversity consequences of climate change. In this vein, Colinet et al. (2015) showed that fluctuating ambient temperatures that remain within tolerant physiological ranges, usually improve performance in insects. Nonetheless, those which cover to extreme temperatures may have both positive impacts, allowing repair of damage accumulated to stressful conditions, or negative impacts from damage during successive exposures.

Biological invasions may interact with global warming, with invasions being favored with the increase in temperatures (Lejeusne et al., 2014; Barahona-Segovia et al., 2016). Zerebecki and Sorte (2011) proposed that invasive species should be less affected by global warming than native ones. For example, native and exotic shrimp respond differentially to increasing temperatures, —the exotic species having better performance at higher temperatures. This hypothesis however was tested for aquatic species, where temperature is less variable than in terrestrial ecosystems. Barahona-Segovia et al. (2016) observed that in native and invasive ladybugs the same hypothesis is not supported, because the native species is as eurythermic as the exotic one.

To predict responses to climate change, physiological ecologists must understand the patterns of thermal variation and the mechanisms by which animals cope with this variation (Burroughs, 2007; Dillon et al., 2010). Within this framework, we experimentally assessed the likely impact of three scenarios of climate change (Burroughs, 2007) on the heat shock response of invasive and native species. Interestingly, we observed that *hsp90* mRNA expression was indeed higher in the invasive species as has been reported in other studies (Henkel et al., 2009; Lockwood et al., 2010; Tomanek and Zuzow, 2010; Zerebecki and Sorte, 2011), but this species did not have a thermal range as wide as the native one (Boher et al., 2010), so we suggest that the biogeographic or evolutionary origin could be playing a role in the heat shock response of these species in different scenarios of climate change. In addition, we observed that the native species did not show *hsp90* mRNA overexpression as a result of our thermal treatments. It seems that, thermically variable as well as constant environments did not represent a stressful condition in species that evolved in harsh environments such as the Andes range. Although the heat shock response is ubiquitous, it varies among species and populations in several ways including the temperature at which HSP synthesis is induced (Somero, 1995).

Comparisons of the heat shock response in species evolutionary adapted to different temperatures, as in our study, have shown that the stress needed to induce HSPs is strongly related to the realized niche of the organism in question (Feder and Hofmann, 1999). For instance, among arctic fish HSPs are induced at around 5°C (Carpenter and Hofmann, 2000) and in thermophilic bacteria at nearly 100°C (Phipps et al., 1993). Among different species of *Drosophila*, it was shown that expression of *hsp70* is lower in lines frequently or continuously exposed to severe stress (Sorensen et al., 1999; Lansing et al., 2000). The interpretation was that the costs of HSPs expression related to fertility/fecundity, development and survival in populations frequently exposed to stress outweighed the benefits and that stress adaptation was achieved through some other means. The same pattern was subsequently found in natural populations of *Drosophila* (Sorensen and Loeschcke, 2001). According to these findings, the adaptive role of HSPs in connection to environmental stress resistance seems to occur during periods of relatively rare, unexpected extreme stress exposures and not during daily environmental fluctuations. On the other hand, the invasive species *D. melanogaster*, shows a marked heat shock response when faced to low mean and low mean-high variance treatments may be because those are stressful conditions from a species originated in tropical environments. Also *D. melanogaster* do present a subtle *hsp90* mRNA overexpression when acclimated at high mean-high variance treatments, again possibly because, although are temperatures within their natural range, variability is perceive as a stress condition.

In conclusion, although the invasive species has the ability to express HSPs over a wider range of thermal conditions than the native species, as have been seen in other invasive-native species comparisons (Henkel et al., 2009; Lockwood et al., 2010; Zerebecki and Sorte, 2011), we suggest that the heat shock response might be also associated with the thermal history of the species, more than a "invasive ecotype" *per-se* (Boher et al., 2012). Indeed, many reports support a lower scope for adaptive evolutionary responses to high temperatures, meaning a more conserved heat tolerance among ectotherms in general (Boher et al., 2010; Bozinovic et al., 2014). Thus, as with upper thermal limits of tolerances, our results suggest that historical biogeography may be an important feature associated with the biochemical response of species under current and future variable climatic scenarios.

AUTHOR CONTRIBUTIONS

Conception and design: FB and FB. Acquisition and analysis: FB and NT. Drafting of the manuscript and revising it: FB, SE, and FB. All authors are approved the final version of the article.

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Age Related Assessment of Sugar and Protein Intake of *Ceratitis capitata* in *ad libitum* Conditions and Modeling Its Relation to Reproduction

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In the inquiry on the age related dietary assessment of an organism, knowledge of the distributional patterns of food intake throughout the entire life span is very important, however, age related nutritional studies often lack robust feeding quantification methods due to their limitations in obtaining short-term food-intake measurements. In this study, we developed and standardized a capillary method allowing precise life-time measurements of food consumption by individual adult medflies, *Ceratitis capitata* (Diptera: Tephritidae), under laboratory conditions. Protein or sugar solutions were offered via capillaries to individual adults for a 5 h interval daily and their consumption was measured, while individuals had lifetime *ad libitum* access to sugar or protein, respectively, in solid form. Daily egg production was also measured. The multivariate data-set (i.e., the age-dependent variations in the amount of sugar and protein ingestion and their relation to egg production) was analyzed using event history charts and 3D interpolation models. Maximum sugar intake was recorded early in adult life; afterwards, ingestion progressively dropped. On the other hand, maximum levels of protein intake were observed at mid-ages; consumption during early and late adult ages was kept at constant levels. During the first 30 days of age, type of diet and sex significantly contributed to the observed difference in diet intake while number of laid eggs varied independently. Male and female adult longevity was differentially affected by diet: protein ingestion extended the lifespan, especially, of males. Smooth surface models revealed a significant relationship between the age dependent dietary intake and reproduction. Both sugar and protein related egg-production have a bell-shaped relationship, and the association between protein and egg-production is better described by a 3D Lorenzian function. Additionally, the proposed 3D interpolation models produced good estimates of egg production and diet intake as affected by age, providing us with a reliable multivariate analytical tool to model nutritional trends in insects, and other organisms, and their

effect upon life history traits. The modeling also strengthened the knowledge that egg production is closely related to protein consumption, as suggested by the shape of the medfly reproduction-response function and its functional relationship to diet intake and age.

Keywords: life time feeding, age related reproduction, Lorentz distribution, capillary feeding, individual medflies

INTRODUCTION

During the past, considerable attention has been devoted to the analysis of food consumption and appetite on groups of animals or on individual organisms (Kogan and Parra, 1981; Cullison, 1982; Gomez-Amaro et al., 2015). However, most of these studies have focused on the total amount of food consumed by the individuals throughout a period or until death, and not at the daily feeding events and individual caloric consumption (Ja et al., 2007). That is, the dissection of ingestion into specific meal parameters such as meal-size and frequency (i.e., prandiology), has been poorly addressed. This level of resolution is in fact of importance in biomedical studies that aim at understanding the physiology and regulation of appetite (Ja et al., 2007). Moreover, from an ecological and demographic perspective, the quantification of diet-size, and frequency of meals through the lifetime of the organism may provide information on changes in feeding patterns throughout aging, allowing us to generate hypothesis on the mechanisms involved in senescence and management of metabolic energy.

Several lines of research on food consumption, physiology, metabolism, and pathology use model-organisms to answer questions with practical implications to human nutrition and disease. Classical model organisms for answering medical questions include laboratory rats and insects. In insects, the vinegar fly, *Drosophila melanogaster*—ironically known as the entomological laboratory "rat"—is mostly used to answer genomic and nutrigenomic questions related to metabolism and diseases (Bharucha, 2009; Ashburner and Bergman, 2016; Ugur et al., 2016). Other insects, like fruit flies (Tephritidae), have also been used as model organisms, but especially in the inquiry of demographic and actuarial questions (Carey and Papadopoulos, 2005). The advantage in the use of insects as model-organisms is related to their short generation time, ability to easily produce large quantities of organisms in a very short period, relative simplicity in their genetic manipulation and development of genetic-lines and close similarity with metabolic pathways of vertebrates, relatively low-costs, and minimal ethical questions of experimentation (Schneider, 2000). Due to these traits, insects can serve as excellent model-organisms to explore questions of nutrition and prandial behavior. Prandiology, refers to the study of specific parameters such as the size and frequency of meals (see Ja et al., 2007).

Methodologies to measure individual intake of nutritional solutions by insects have been proposed. These include the Capillary Feeder (CAFE) method (Ja et al., 2007) used with *D. melanogaster*, the Phagostimulation Unit Bioassay (PUB; Nestel et al., 2004) used with tephritid fruit flies, and the use of pipette tips as dispensers of food solution used also with tephritids (Fanson et al., 2009). These methods have been used to answer questions such as the toxic effect and kinetics of ingesting stomach poison in fruit flies (Nestel et al., 2004), the prandiology of *D. melanogaster* (Ja et al., 2007), the effects of the "social context" on intake and energy management in tephritids (Zur et al., 2009) and the effect of dietary restriction on reproduction and survival of Tephritidae fruit flies (Fanson et al., 2009). An interesting aspect of prandiology investigation is the possibility of linking individual intake with physiological performance, reproduction, and longevity. While Fanson et al. (2009) linked intake with reproduction and survival on the Queensland fruit fly, their system provided intake information at a 4 day intervals. On the other hand, the PUB system has technical limitations to be able to concomitantly measure intake and reproduction. The only alternative to be able to provide this type of information is the use of the CAFE system with the specific aim of also measuring individual nutrition and reproductive effects. The intentions of the present study were, thus, to explore the use of CAFE as a prandiology system suitable for providing information on individual daily intake of nutrients and its effect on reproduction and longevity. We used the Mediterranean fruit fly (medfly), *Ceratitis capitata* (Diptera: Tephritidae), as a model organism to demonstrate the ability of the CAFE system to provide prandiology information at a resolution of the individual organism and at relatively short time intervals. Former quantitative methods have focused on measuring feeding of insects on the simultaneous combinations of different ratios of protein and sugar (Nestel et al., 2016). In the current study, however, one source of food is given *ad libitum* and in solid form, while the other is given for a specific time-period in liquid form and measured, gaining discrete measurements on the consumed amount of liquid food source in the presence of a second nutrient source. We also show the ability of our system to provide individual information on reproduction, longevity, and nutrition, and mathematically and statistically link between the different measured factors at the individual level. Our objectives were: to develop and standardize a reliable method for prandiology studies in fruit flies and to apply modern multivariate analysis techniques to the results, ultimately providing a useful framework for future prandiology studies.

MATERIALS AND METHODS

Experimental Flies and Laboratory Conditions

The medflies used in the experiments were of the F_2–F_8 laboratory generation established from pupae collected from

naturally-infested figs (*Ficus carica* L.), pears (*Pyrus communis* L.), apricots (*Prunus armeniaca* L.), and peaches (*P. persica* L.) around Thessaloniki (northern Greece, 40°31′N, 22°58′E). Specifically, we used flies of the F2–F6 laboratory generations for standardizing the methodology (see below), whereas the main life time experiments were performed using adults of the F7–F8 generations. Collected pupae were mixed to create batches of ∼300 individuals each and placed in 9 cm diameter plastic Petri dishes until adult emergence. The colonies were maintained in wooden, nylon screened, holding cages (30 by 30 by 30 cm) and provided with food (a mixture of yeast hydrolysate and sugar in a ratio of 1:4 in the form of solidified droplets), water and oviposition substrates. Each oviposition substrate consisted of a red, plastic, hollow hemisphere (5 cm diameter) with about 100 evenly distributed holes of 0.7 mm diameter through which females laid their eggs. The base of each dome was fitted into a hole made on the lid of a plastic Petri dish. To stimulate oviposition, 5 mL of freshly-squeezed orange juice were placed into the Petri dish. Eggs were collected with a fine brush and placed on standard larval medium for immature development. Details regarding the composition as well as the preparation procedures of larval diet are described by Boller (1985). The experiments were conducted in the laboratory of Applied Zoology and Parasitology of the Aristotle University of Thessaloniki, Greece, under a 14:10 h light: dark cycle at 25 ± 2°C and 55 ± 10% relative humidity.

General Methodology to Assess Food Ingestion and Egg-Laying

The method we developed is a modification of the CAFE bioassay proposed by Ja et al. (2007). Medflies were placed inside 400 ml volume individual plastic cages. Tested solutions were provided in a glass capillary tube (Cat. No. 29 000 00; Marienfeld-Superior, Germany), with one end of the capillary inserted inside the cage and accessible to the flies. Water was provided *ad libitum*. In addition to water, we placed complementary solid food inside the cage. Solid food consisted of either sugar, if we were measuring solutions of protein hydrolysate in the capillary, or solid protein hydrolysate, if we were measuring solutions of sucrose in the capillary. This experiment allowed quantification of ingestion of a nutrient while the other nutrient was provided *ad libitum*. In the experiments where oviposition was measured, the cages also included a device for egg-laying, which allowed recording of the amount of eggs laid during the experimental period. A detailed drawing of the cage and the capillary feeding mechanism is shown in **Figure 1**.

Prior experimental experience with the CAFE method has shown that to achieve reliable and high precision measurements it is required a low evaporation through the capillaries providing the food solution. Therefore, it is necessary to sustain a rather humid environment during the time capillaries are inserted in the cages or otherwise evaporation of capillary solution is relatively high. In our experiments, to reduce evaporation and prevent dripping, and thus increase the accuracy of our method, a small quantity of paraffin oil was added in the upper extreme of the capillary tube. In addition to that we conducted the experiments inside the "Humidity Preservation Chamber" (HPC) described in the next paragraph. In each HPC we established five individual cages, plus a control cage which did not contain any fly and served to measure evaporation from the capillaries. The descent of the meniscus of the food solution in the capillaries as the insects were feeding allowed continuous, unambiguous measurement of feeding events and diet consumption. This measurement was corrected by subtracting the amount evaporated from the capillary in the control cage. The remaining amount was, thus, recorded as the volume of solution consumed by an insect during the 5 h of exposure.

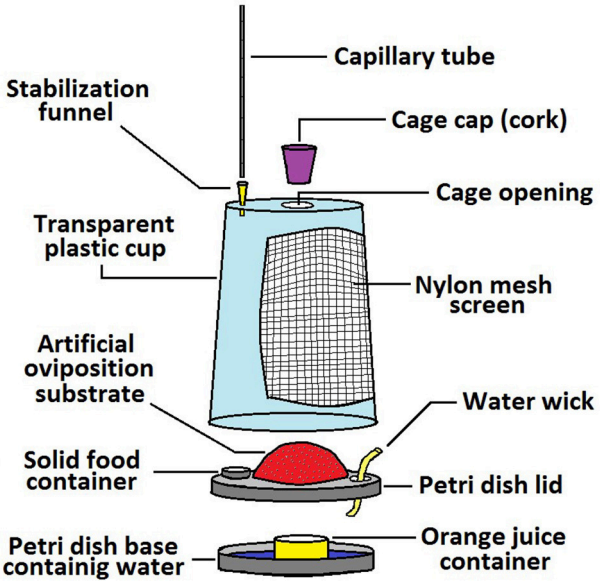

FIGURE 1 | Schematic diagram of the individual cage and capillary tube used to measure the lifetime daily intake of liquid food and oviposition by *C. capitata*. The cage consisted of an inverted transparent 400 ml volume plastic drinking cup (12 cm high, 6.5 cm base diameter, and 9 cm top diameter). The bottom part of the cage was the lid of a 9 cm-diameter plastic Petri dish attached to the open large surface of the inverted cup. A lateral 5 × 8 cm window covered with nylon-mesh-screen was perforated on the cup's side for ventilation while a 2 cm-diameter opening was cut on cup's top center serving as an entrance for the adult medflies and a cork was used as a cap. Sugar or protein hydrolysate was provided as a water solution by the capillary tube at the top of the cage whereas solid food was provided in a small container at the base of the cage. Peripherally of the top smaller surface of the inverted plastic cup (2–3 mm from the edge) a hole was made and a cut pipette tip (3 cm height) was set stabilizing the vertically inserted glass capillary tube. The bottom 1 mm end of the capillary projected inside the cage. To decrease evaporation, the top part of the capillary was sealed with a tiny drop of paraffin oil. A 5 cm diameter circular hole was cut in the middle of the petri dish lid forming the base of the cage and a hollowed plastic dome perforated with small holes serving as an oviposition substrate was attached. The petri dish base under the dome contained water and a smaller cylindrical cup filled with 5 ml of fresh orange juice (plus 1% sorbic acid) serving as an oviposition stimulant. The orange juice was replaced every 2 days. Individual cages for males lacked both the oviposition substrate and the orange juice container.

Effect of the High Relative Humidity on Egg Production

It is known that high humidity levels may affect oogenesis in fruit flies (Broufas et al., 2009). To assess a possible negative effect of the required high relative humidity in our method on egg production, we conducted an additional experiment. Females were placed in four 25 by 25 by 35 cm cages in groups of 125 per cage. The cages contained water and ample sugar and protein (mixed in a ratio of 3:1, respectively) in solid form. In two of these large cages (referred to as HPCs), relative humidity was maintained high for 5 h every day (from 12:00 to 17:00 h) using water soaked Wettex and plastic that covered the screen openings. The other two cages remained uncovered serving as controls; relative humidity in these cages was low, around 40%, and at the same level as in the experimental room. Daily, and for eight consecutive days, 10 females from each of the two HPCs and the control cages, totaling 160 females for the treatment and 160 for the control, were sacrificed in alcohol and their ovaries dissected to assess the number of mature eggs.

Effect of Nutrient Concentration on Food Intake and Oviposition

To select a concentration of liquid food and to "calibrate" our method we used only females. We tested three different concentrations (5, 10, or 20% w/v) of liquid sugar and liquid protein. When testing liquid sugar, protein was provided *ad libitum*, whereas, when testing liquid protein solid sucrose was provided *ad libitum*. We used 20 individual female cages per concentration. Five individual cages hosting females, and one control cage without a female serving to measure evaporation, were placed inside an HPC. During 15 days, food was provided to these females for 5 h per day, from 12:00 to 17:00 h, and consumption was measured at the end of the 5 h period. The aim of the experiment was to find a concentration that corresponded to a volume of consumption large enough to be measured reliably, while also providing the females with adequate nutrients to mature their eggs. To measure the reproductive potential associated with diet type, after 15 days all females from the different treatment concentrations were sacrificed in alcohol and their ovaries were dissected to quantify mature eggs.

Life Time Consumption Patterns of Protein and Sugar, and Related Egg-Laying Patterns

The previous experiments provided us with the basis to establish a long-term experiment to measure food intake in male and female medflies and female egg-laying patterns. Males and females were maintained individually (as described above) in the individual cages placed inside HPCs. Thirty individual males were exposed for 5 h daily to a solution of 20% sucrose in capillaries to measure daily intake, while they were allowed free access to protein hydrolysate *ad libitum*. Simultaneously, 30 individual cages with single males were exposed to the opposite condition: 20% protein hydrolysate solution provided to them in capillaries for 5 h. daily, while sucrose was provided *ad libitum*. Simultaneously, two groups of 30 individual female cages were also established in a similar fashion to the ones described for males, and intake of 20% protein hydrolysate or sucrose was measured daily after 5 h as described for males. In contrast to males, however, females could mate, and the eggs deposited in the egg-laying devices were collected daily. To allow mating and fertilization of eggs, three adult males which had been kept separately but were of the same age as the females and were well fed with solid protein hydrolysate to assure proper sexual development, were introduced into the individual female cages at the age of 10 days and removed after 5 days, at the age of 15 days old. The males were also removed from the cage for 5 h daily, during the time of capillary feeding by the female, and reintroduced after the capillary was removed and female intake quantified. The same "mating" procedure was repeated between days 30 and 35 of adult age. This experimental design with female medflies allowed us to measure daily intake of the tested solutions and egg production during a long-term period (for more than 40 days in most cases and in several cases for up to 60 days), until all individual females died. Male individual consumption was followed also until the last individual died, which extended for more than 150 days.

Data Analysis and Modeling of Age Related Diet Intake

Revealing potential interrelationship between medfly diet intake, egg production, and longevity is a complex—even though challenging—task, mainly due to the non-linear nature of the egg production and survivorship curves. Consequently, we used different modeling approaches to get a consensus on their interrelationships. We implemented event history charts, ANOVA and repeated measure analysis, as well as 3D non-parametric and parametric models as described below.

First, the description of daily intake and egg-production throughout the adult life of individual insects was conducted using event history charts (Carey et al., 1998). Event history charts allow plotting the daily consumption or egg-production of the individual fly throughout life, providing a visual method to explore intensity in food consumption and egg-laying as related to adult age.

Second, all data were further evaluated under the assumptions of normality and differences in solution intake between concentrations of protein and sucrose were inferred using typical parametric ANOVA and the non-parametric Kruskal-Wallis method (SPSS, 2013). The effect of adult age, diet type and sex until day 30, as well as their interactions, were inferred using repeated measures ANOVA (Girden, 1992). The between subject effects were tested using Pillai's trace while the assumption of Sphericity was tested using Mauchly's test ($a = 0.05$). Within subject effects was performed when sphericity was not assumed using the Greenhouse and Geisser (1959) and Huynh and Feldt (1976) procedures to correct the degrees of freedom for the F-distribution. The effect of diet on medfly longevity and hazard rate was compared using Kaplan Meier survival analysis followed by log-rank tests ($a = 0.05$).

Finally, the relationship between age, dietary intake, and egg production was explored, until all individuals died, using

the non-parametric interpolation method LOESS (LOcally WEighted Scatter-plot Smoother, Cleveland, 1979; Cleveland and Devlin, 1988) and a parametric non-linear regression based on a 3D Lorenzian type distribution function (Devanne et al., 2006).

The LOESS procedure allows great flexibility because no assumptions about the parametric form of the regression surface are needed and is suitable when there are outliers in the data and a robust fitting method is necessary (Jacoby, 2000). In brief, the main features of the LOESS procedure assumes that for $i = 1,....,n$ the ith measurement y_i of the response y and the corresponding measurement x_i of the vector x of p predictors are related by: $y_i = g(x_i) + \varepsilon_i$. Where g is a predefined smooth function (i.e., local polynomial) and ε_i is a random error (Jacoby, 2000). The function g can be locally approximated by the value of a regression function, of some specified parametric class and the method can further be extended in the space domain (i.e., 3D, Lekien and Mardsen, 2005). The size of the neighborhoods for local regression is further defined by a smoothing parameter, q: $(\lambda + 1)/n < q < 1$. Where λ is the degree of the local polynomial for sample n and regressed using a weight function, w, to perform a least square fit (SAS Institute Inc., 1999).

In this study, we have used a standard tricube weight function of the form: $w(x_i) = \left(1 - |x_i|^3\right)^3$, for $|x_i| < 1$ and $w(x_i) = 0$, for $|x_i| > 1$ and a local polynomial regression of degree 1. Moreover, sampling was performed for a smoothness parameter $q = 0.1$ for the protein intake data and for $q = 0.2$ for the sucrose solutions, respectively. "Lower" parameter values were also tested but proved to be sensitive to extreme sampling error since they pushed the highest point of the 3D mesh and caused in some cases overestimations.

Although the non-parametric interpolation method is very flexible for modeling complex processes, for which no theoretical model exists, it does not provide any abstract information on the significant interaction between variables. Therefore, as a complement, we have decided to run 3D parametric regression mesh model, using ordinal regressions based on a Lorenzian type of normal distribution. The model is given as follows (Devanne et al., 2006):

$$f(x,y) = \frac{\alpha}{[(1 + \left(\frac{x-x0}{\beta}\right)^2] \cdot [1 + \left(\frac{y-y0}{\gamma}\right)^2]}.$$

The above function has five parameters $x0$, $y0$, α, β, and γ, and which can be interpreted to a certain degree. Parameter α represents the estimated amplitude of the maximal egg production that is located at coordinates $x0$ and $y0$. Moreover, β and γ reflect the slope and width of the egg production peak in relation to diet intake and age, respectively.

The slope parameters α and β obtained from the Lorentzian function, as well as the differences between the coordinates ($x0$, $y0$), were compared using one-way ANOVA and the Student's t-test for 0.05 significance level. The interpolation models were generated using Macro Recorder script modules and the Interactive Development Environment (IDE) in SigmaPlot (SigmaPlot® 8.0 Programming Guide)[1].

RESULTS

Standardization of the Methodology

Relative humidity inside the HPC strongly contrasted with the relative humidity measured in the control cages and in the laboratory environment (**Figure 2A**). While the relative humidity inside the HPC was kept around 80% during the afternoon hours (12:00–17:00 h), the ambient relative humidity in the laboratory during that period of the day was around 40%. The effect of the two different humidity environments did not affect the overall egg-laying potential, as reflected by the similarities in oogenesis of female medflies in the two types of environments (**Figure 2B**;

[1]SigmaPlot® 8.0. Programming Guide. Copyright © 2002 by SPSS Inc.

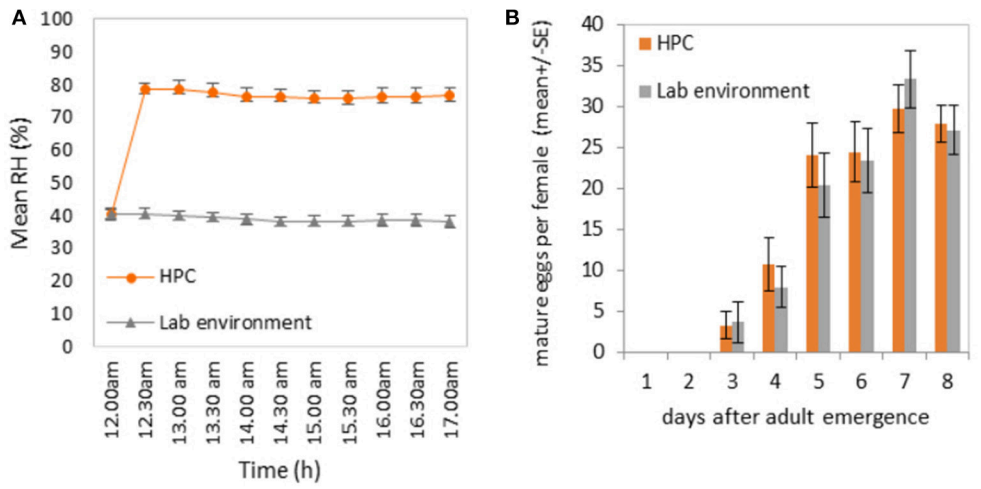

FIGURE 2 | (A) Relative humidity (RH) levels inside the "Humidity Preservation Chambers" (HPC) and in the laboratory environment (25 ± 2°C). Measures were performed every half hour for a total of 5 h (from 12:00 to 17:00). **(B)** Effect of the two relative humidity environments on the number of ovarioles per fruit fly.

$t = 0.436$, $df = 38$, $p = 0.66$). These results strengthened the use of the HPC to constantly maintain a high level of humidity during the long-term food-intake and egg-production study, thus avoiding undesirable evaporation of the liquid food.

An important aspect of our study was to estimate the "optimal" concentration of nutrient solutions that will maintain good levels of nutrition and egg-production in female medflies, but will also allow quantifying nutrient uptake with a large degree of certainty and reliability. **Figure 3** shows both the cumulative amount of nutrient ingested during 15 days by individual females as affected by the concentration of sucrose and protein hydrolysate solutions and the effect of food concentration on the number of mature ovarioles developed by single females during the 15 days of the experiment. Both protein hydrolysate and sucrose ingested amounts were significantly larger in the 20% solutions than in the others ($F = 12.76$, $df = 59$, $p > 0.01$ and $F = 251.13$, $df = 59$, $p < 0.01$ for sucrose and protein hydrolysate solutions, respectively), and the number of ovarioles was significantly favored by the 20% solutions of both nutrients ($F = 5.34$, $df = 59$, $p < 0.01$ and $F = 38.65$, $df = 59$, $p < 0.01$ for sucrose and protein hydrolysate solutions, respectively). These results, thus, pointed at the 20% solutions as appropriate for the long-term intake and egg-laying study.

Patterns of Nutrient Uptake by Male and Female Medflies and Female Egg-Laying

Age-specific intake of nutrients by adult female and male medfly is shown in the event history charts in **Figure 4**. Both, males and females, ingest relatively large amounts of sucrose during the first days of adult-life (first 10 days). Sucrose consumption in females then drops and is maintained relatively low until death. Males, in contrast, show an intermittent consumption of sucrose solutions, which is marked by a drop in adult ages 10–20 days, and increased sucrose intake in mid-life and advanced ages (30–100 days old).

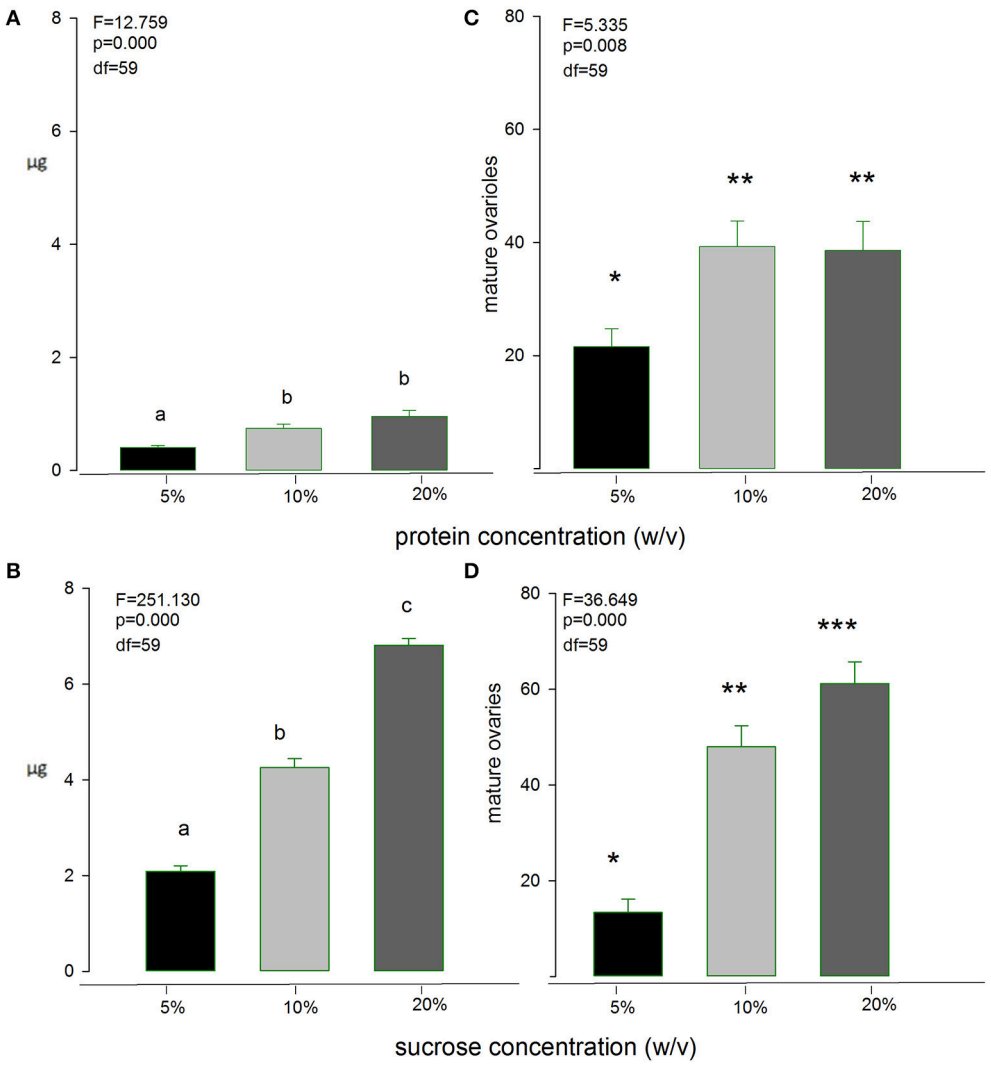

FIGURE 3 | Intake of Protein-hydrolysate solution (A) and sugar **(B)** by medfly females as affected by the concentration of the solution, and effect of solution concentration on maturation of ovarioles **(C,D)**. Bars with different symbols or letters are significantly different at 0.05 level.

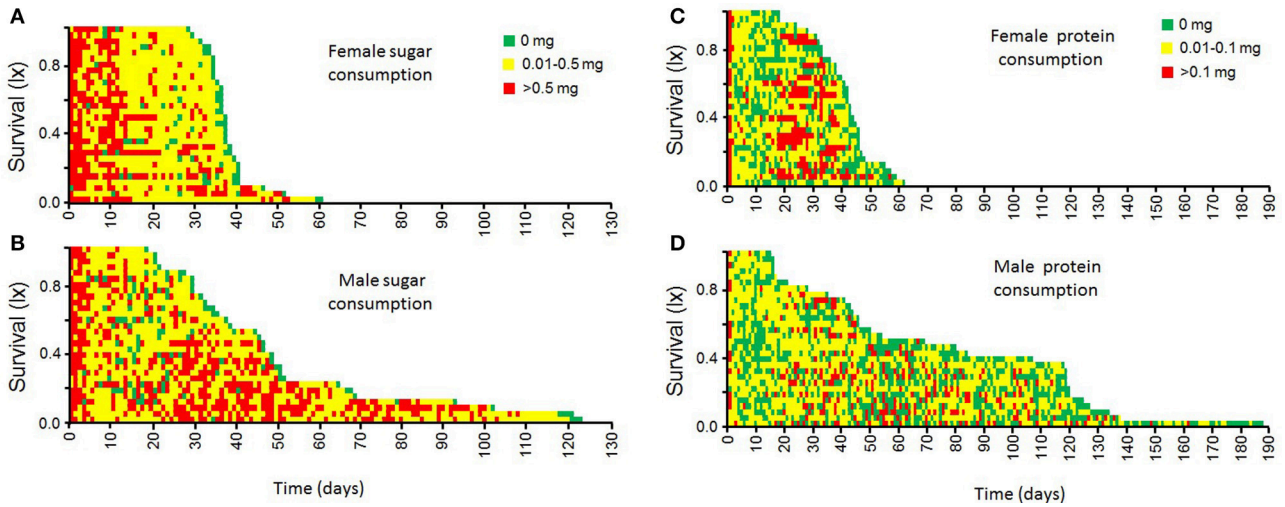

FIGURE 4 | Individual patterns of sucrose (A,B) and protein hydrolysate **(C,D)** consumption by female and male medflies throughout their life.

Female medflies tend to have an initial relatively large intake of protein hydrolysate during the first 2 days of adult life, then it drops and increases toward mid-life (20–40 days old), when the first batch of eggs are being laid (**Figures 4**, **5**). Males, in contrast, showed a more stable and constant intake of moderate levels of protein hydrolysate throughout their adult life-span (**Figure 4**).

Egg-laying patterns as affected by the solution of nutrient provided to the female are shown in **Figure 5**. Females fed with protein solutions produced similar numbers of eggs per day compared to females fed with sucrose solutions. The main wave of egg production coincides with the patterns of intake of protein hydrolysate solution by female flies (**Figure 4**). Apparently, diet type did not significantly affect the life time egg production ($F = 1.63$, $df = 1$, $p = 0.21$).

Average individual intake of sucrose during the first 30 days did not differ between male and female medflies. In contrast, individual intake of protein hydrolysate during the first 30 days of adult life was significantly higher in female than in male medflies. Both males and females consumed significant higher amounts of sucrose than protein hydrolysate (**Figure 6A**; $F = 469.972$, $df = 119$, $p < 0.005$). Total nutrient consumption by males and females fed with sucrose or protein hydrolysate throughout their lifespan was also compared and it was found that it did not differ between male and female medflies ($p > 0.05$). Additionally, both sexes consumed significant higher amounts of sucrose than protein hydrolysate (**Figure 6B**; $F = 48.217$, $df = 119$, $p < 0.005$).

The Log Rank (Mantel-Cox) test revealed significant differences among the four adult cohorts ($X^2 = 29.128$, $df = 3$, and $p < 0.005$). Particularly, male longevity was longer than that of females (**Table 1**). Furthermore, the ingestion of protein hydrolysate solutions significantly expanded longevity, at least in male medflies.

Patterns in nutrient consumption by males and females throughout their lifespan were explored by contrasting the average consumption between the two sexes. **Figure 7** shows the relative female/male diet consumption of protein hydrolyzate and sucrose until day 60 of adult life. During the first 30 days, consumption of sucrose and protein hydrolyzate is relatively similar in the two sexes. However, after day 30 of adult life, females tend to double, and even triplicate, the ingestion of protein compared to males (**Figure 7**). Consumption of protein by females oscillates in time with peaks of consumption (of up to 3.5 times that observed in males) with a periodicity of a few days between peaks. Consumption of the sugar solution after day 30 is kept in average at around 1, with slight oscillations showing higher consumption of sugar by male medflies. However, correlations between relative diet consumptions and egg productions for individuals feed with sucrose or protein solution were not significant ($p > 0.05$).

Non-linear Modeling Egg-Laying as a Function of Nutrient Uptake and Age

Non-linear modeling provides a surface-plot analysis methodology to link intake with egg-production and age, which is not possible using other types of analysis. Age related egg-laying progression differs between medflies fed with sucrose and protein hydrolysate (**Figures 8A,B**). Large number of laid eggs is related both to a relatively large consumption of sucrose and a relatively intermediate intake level of proteins. The highest level of egg production was observed at the age of 40–50 days when medflies consumed between 0.5 and 0.6 μg sucrose and between 0.12 and 0.17 μg of protein hydrolysate. Egg-laying, thus, is maximal at mid-life of the cohorts and appears to be bell shaped with pronounced tails. Moreover, fitting of the Lorentzian function to the data points allowed calculation of the coordinates of the optimal point of egg production (i.e., parameters *x0* and *y0*). Additionally, the two slope parameters, α and β, which give an indication of the shape of data distribution were significant in most cases, although the coefficient of determination was lower (0.489) for the sucrose egg production data compared to the protein (0.903).

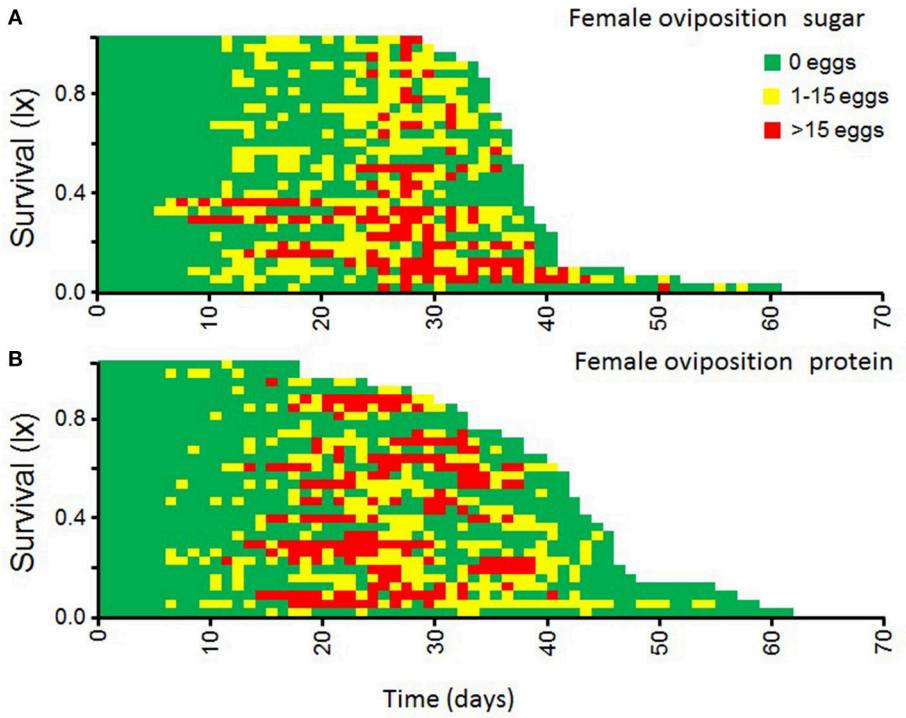

FIGURE 5 | Patterns of individual egg-laying in female medflies fed sucrose solutions (A) and protein **(B)** hydrolysate solutions.

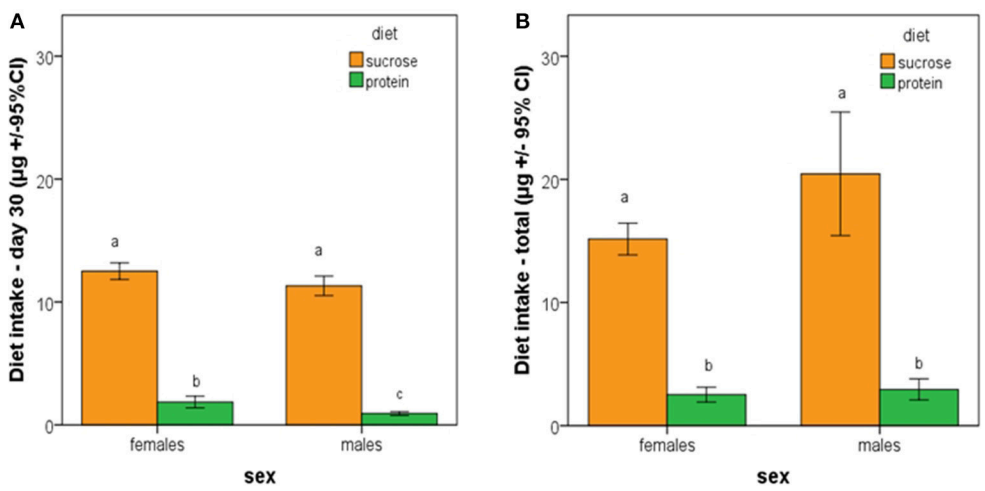

FIGURE 6 | Average individual cumulative ingestion of sucrose and protein hydrolysate by female and male medflies during (A) the first 30 days of adult life and **(B)** during total adult life. Bars with different letters are significantly different at 0.05 level.

Moreover, the respective ANOVA model accounted for most of the data variability and *F*-statistic was significant for both the sucrose egg production data ($F = 13.205$, $df = 59$, $p < 0.000$) and the protein data ($F = 129.401$, $df = 59$, $p < 0.000$), respectively.

Consequently, both patterns could be described well by the Lorenzian type regression functions. In addition, the regression models for all variables were found to be significant (**Tables 2**, **3**). **Figures 8C,D** show the fitting of the data to the models. Indeed, egg production data, and especially that related to protein hydrolysate intake, were soundly described by the Lorenzian type normal distribution function, with 95% of the variability accounted by the data (**Tables 2, 3**). The parameter terms were found significant in most cases ($p < 0.05$) and their influence was found to be the most important in ANOVA model calculations.

TABLE 1 | Survival time of male and female medfly cohorts fed with sugars and proteins.

Diet	Gender	Mean			Median		
		Estimate ± *SE*	95% confidence interval		Estimate ± *SE*	95% confidence interval	
			CI low	CI high		CI low	CI high
Sugar	Female	38.935 ± 1.133	36.715	41.156	38.000 ± 0.556	36.909	39.091
	Male	50.833 ± 5.031	40.973	60.694	46.000 ± 4.930	36.338	55.662
Protein	Female	40.057 ± 1.925	36.285	43.829	43.000 ± 1.932	39.213	46.787
	Male	75.037 ± 9.296	56.817	93.257	55.000 ± 19.905	15.986	94.014
Overall		50.081 ± 2.738	44.715	55.448	42.000 ± 1.201	39.647	44.353

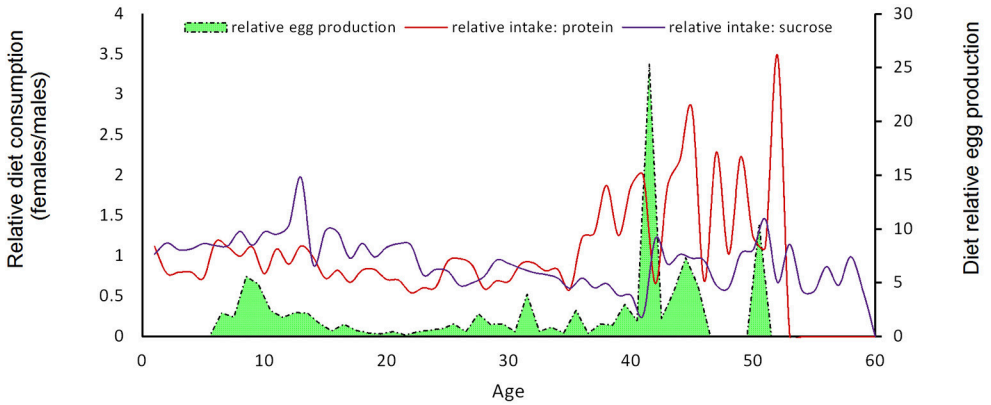

FIGURE 7 | Female/male medfly relative diet ingestion of sugars and proteins (in μg) until day 60 and relative egg production (eggs with sugar intake/eggs with protein intake).

DISCUSSION

In this study, we developed and standardized a methodology suitable for measuring individual daily food ingestion of the adult *C. capitata* throughout life span. This method enables high precision measuring of daily food intake while at the same time allows to relate intake to individual egg production in fruit flies, which has rarely been concomitantly measured. Moreover, in contrast to other studies, which explore diet consumption during a short period of the insect's life, the current work screens diet consumption until all individuals have died. This is a major advantage because the influence of nutrition may affect not only the metabolic needs and reproductive potential during early ages but also have differential effects throughout the entire lifespan.

One important novelty of the current experimental set up, compared to other studies, is that medflies had *ad libitum* access to solid diet while presented to a liquid solution of food. This exposure of flies to two different sources of food allowed us to explore intake of one of the nutritional food sources in the presence of an alternative food source. This provides information on the timing and need of specific nutritional elements during development and lifespan of the insects. For example, the results, confirmed that *ad libitum* laboratory reared medfly has an ability to consume high energy sources in the form of solutions during a range of its lifespan which coincides with a high reproductive budged. In fact, this type of experimental setting could provide an elegant framework to investigate the prandiology of specific nutrients, such as single amino acids, or the phagostimulatory properties, and reproductive effects, of secondary plant metabolites or other ingested substances, such as the parapheromone methyl eugenol (Tan et al., 2014).

The CAFE capillary method developed by Ja et al. (2007) for *Drosophila*, despite being an effective system to measure intake, may substantially reduce egg-laying and life span compared to techniques which provide food in agar-gelled medium (Bass et al., 2007; Ja et al., 2007; Wong et al., 2009). In the case of our study, with the modified CAFE system, egg-laying and life-span were comparable to studies using the provision of add-libitum food sources (Carey, 2003). Moreover, compared to indirect measures, which are usually based on regular administration of radioactively-labeled nutrients after transferring flies to food (King and Wilson, 1955; Carvalho et al., 2005), our system provides undisturbed feeding conditions and allows measures of intake and egg-laying for long-periods of time without possible adverse effects on reproduction.

A limitation of our method is that when liquid diet is provided, older adults may not be able to have free access to the food solution provided in capillaries positioned in the roof of the individual cages. As we have shown in an earlier study adult medflies at older ages lose capacity to climb on cage walls and

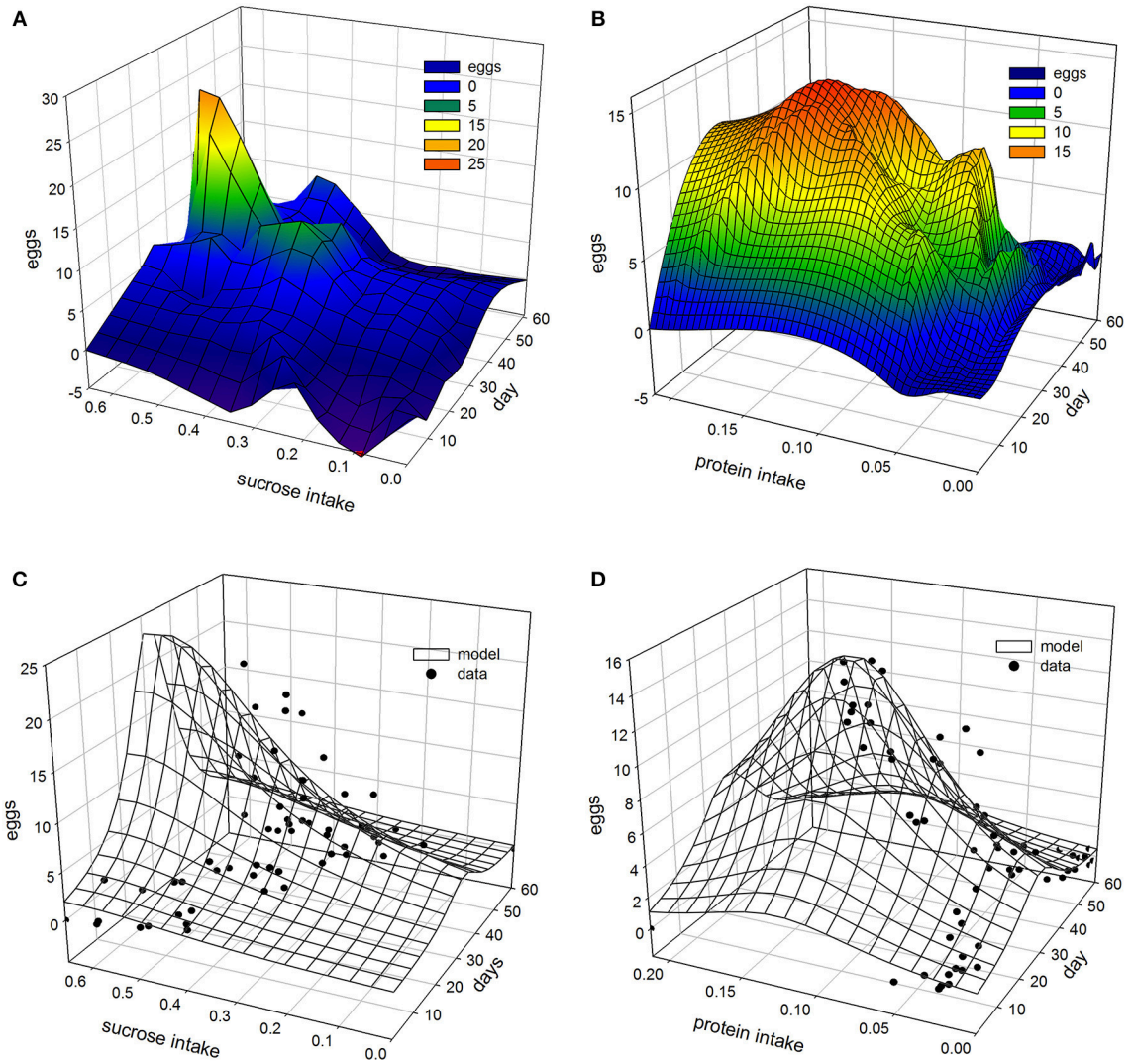

FIGURE 8 | Egg production as a function of diet intake using 3 days interpolation LOESS method for sugar (A) and protein **(B)** and 3D Lorentzian regression model for sugar **(C)** and protein **(D)**.

spend a progressively increasing amount of time in a supine position (Papadopoulos et al., 2002). Apparently, these senescent adults have limited food location capacity. Nonetheless, this does not affect both conclusions regarding food consumption during the active life span of the individuals and the aim of the current paper, which was to demonstrate and test a prandiology system for life time assessment of food intake. Simple adjustments, regarding positioning of the capillaries within the individual cage, may address this issue. It should also be noted that liquid diet influences food intake and energy balance in an organism in a very different and sometimes complex way relative to a comparable solid diet (Simpson and Raubenheimer, 1999; DiMeglio and Mattes, 2000). This is further supported by the burst in egg production observed in our study after day 40 in females eating less sugar, but more protein (see **Figure 7**). Even though no statistically significantly correlation exists, this outcome supports the notion of protein as a booster of egg production and the possibility that protein provided in liquid form enables a higher egg-production yield than solid protein provided *ad libitum*. We therefore suggest that researchers with different objectives than ours should consider providing food in the same form, either only liquid or only solid.

Our results suggest that the high relative humidity (i.e., HPC vs. the lab environment) did not affect the overall egg-laying potential, as reflected by the similarities in the number of ovarioles in female ovaries. In general, tephritids are mainly tropical or subtropical in origin and need hot, damp (50–80% relative humidity) conditions for optimum egg production (Fay, 1989). For example, in *Bactrocera oleae* low relative humidity suppressed ovarian maturation, egg production, and longevity whereas high relative humidity in the range from 55 to 75% favored them (Broufas et al., 2009).

TABLE 2A | Non-linear 3D model regression statistics to describe the relation between sugar intake, egg production, and age in the medfly.

Model	*R*	R^2	Adjusted R^2	*SE* of estimate
3D, Lorentzian	0.6999	0.4899	0.4528	3.7487

TABLE 2B | Model parameters.

Coefficient		*SE*	*t*	*p*-value
*x*0	32.0000	1.1310	28.2947	<0.0001
*y*0	0.6276	0.2476	2.5341	0.0142
α	24.0087	17.9864	1.3348	0.1874
β	9.2432	1.7346	5.3288	<0.0001
γ	0.2749	0.1077	2.5531	0.0135

TABLE 2C | Analysis of variance: corrected for the mean of the observations.

	df	SS	MS	*F*	*p*-value
Regression	4	742.2742	185.5686	13.2055	<0.0001
Residual	55	772.8826	14.0524		
Total	59	1515.1569	25.6806		

TABLE 3A | Non-linear 3D model regression statistics to describe the relation between protein intake, egg production, and age in the medfly.

Model	*R*	R^2	Adjusted R^2	*SE* of estimate
3D, Lorentzian	0.9508	0.9039	0.8970	1.5619

TABLE 3B | Model parameters.

Coefficient		*SE*	*t*	*p*-value
*x*0	28.3359	0.6892	41.1140	<0.0001
*y*0	0.1258	0.0134	9.3826	<0.0001
α	14.8297	1.0309	14.3857	<0.0001
β	12.7151	1.4940	8.5106	<0.0001
γ	0.0725	0.0137	5.2809	<0.0001

TABLE 3C | Analysis of variance: corrected for the mean of the observations.

	df	SS	MS	*F*	*p*-value
Regression	4	1262.7094	315.6773	129.4014	<0.0001
Residual	55	134.1736	2.4395		
Total	59	1396.8830	23.6760		

Regardless of diet type males in this experiment survived significantly longer than females. Similar results have been reported by Diamantidis et al. (2009) testing six different medfly biotypes. As far as the effects of protein availability on male and female longevity are concerned, the results are rather controversial. For example, Plácido-Silva et al. (2006) and Chang et al. (2001), did not observe any differences between males and females when fed with similar amounts of protein diet. However, in a seminal paper, Muller et al. (1997) have proven that life expectancy of both male and female medflies is reduced if they are deprived from protein and that the reduction is far greater in females resulting in a reversal of female advantage in life expectancy. It seems that external factors, such as dietary manipulations may alter life span advantage of the one over the other sex (Carey, 2003).

In the current experiments females were mated but males were not. Mating is known to induce cost in terms of life span reduction in both female and male medflies (Papadopoulos et al., 2010; Papanastasiou et al., 2013). Similar results regarding the cost of mating have also been reported for the olive fly (Gerofotis et al., 2015). This lack of mating cost may in part account for the substantially larger lifespan in males relative to females observed in our study. It is well appreciated in fruit fly research that females need high amounts of protein for egg laying and apparently sugar to fulfill energy requirements induced by other behavioral activities. Energy requirements are minimal in the small individual cages since foraging demands (search for oviposition sites and food) are essentially absent. On the other hand, it has been shown recently that male medflies require increased amounts of both sugar and protein intake to perform the various energetically demanding sexual calling activities of pheromone production, wing vibration, etc. (Papadopoulos et al., 1998; Papanastasiou et al., 2013). Female egg laying and male sexual activities are both energetically and dietary demanding and may thus trade-off with lifespan in ways that result in differences between the two sexes. In our case, differences are more profound in males because the overall energy taken from food can be partitioned only in lifespan, given that males in individual cages were prevented from any other energetically costly activity (e.g., aggressive behavior to other rivals). In females, the overall energy was partitioned between lifespan and egg-laying and thus the magnitude of the effects on each trait is expected to be different than in males and much harder to detect. However, the current experimental set up does not test for possible trade-offs and neither dietary nor calorie restriction or other effects.

Regarding the different modeling approaches, which were applied to the data, it is important to outline that each has specific advantages and limitations and thus were all used to find a consensus on the interrelations between dietary intake, egg production and longevity. The event history charts, for example, have the advantage of discovering a specific behavioral pattern by age, yet are mostly descriptive. Furthermore, the use of typical statistical models, such as one-way ANOVA, does not account for the effects of age in the analysis (Field, 2011). On the other hand, the repeated measures analysis, although it provides a good way of statistically assessing the change of a variable over time, it is strongly affected when within subject factors exceed more than two levels and is vulnerable to effects from missing values (i.e., mortality at advantaged ages). In respect to the non-parametric LOESS method, the biggest advantage is that it does not require the specification of a function to fit a model to all

the sample data and the analyst must provide simply the degree of polynomial and smoothing parameter value. However, it's rather difficult to statistically evaluate the functional dependence between the variables of interest. Ultimately, the parametric 3D surface models may be viewed as a way of merging the observed actual event history data of a cohort with a statistical model, although there are cases where it's quite difficult to obtain a suitable model that fits over all data set. For example, the Lorentzian like model fits remarkably better to the protein data compared to the sucrose data density in which both slope parameters, α and β, where significant and gave an indication of the egg production steepness of the 3D surfaces in respect to diet intake and aging.

Thus, the 3D interpolation models produced good estimates of egg production and diet intake as affected by age, providing us with a reliable multivariate analytical tool to model nutritional trends in insects, and other organisms, and their effect upon life history traits. To our knowledge, this modeling has seldom been used to relate individual fruit fly intake with egg-laying, providing us with an excellent approach in prandiology studies, not only of insects but for other organisms as well. This type of modeling allowed us to strengthen the knowledge that egg production is closely related to diet consumption, as suggested by the shape of the medfly reproduction-response function and its functional relationship to diet intake and age. It also provided day by day information of this biological relationship. We believe that its use may allow more insights into the functional relationships between nutrition and other biological traits in higher organisms.

AUTHOR CONTRIBUTIONS

NK, NP, DN, and CI conceived and designed experiments; CT and CI performed experiments; PD, CT, NK, and CI prepared figures and tables; PD, CI, DN, NK, NP, and DK analyzed and interpreted results, and wrote the paper; NK edited and revised the manuscript.

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Silencing of *CYP6* and *APN* Genes Affects the Growth and Development of Rice Yellow Stem Borer, *Scirpophaga incertulas*

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RNAi is a powerful tool to target the insect genes involved in host-pest interactions. Key insect genes are the choice for silencing to achieve pest derived resistance where resistance genes are not available in gene pool of host plant. In this study, an attempt was made to determine the effect of dsRNA designed from two genes *Cytochrome P450 derivative* (*CYP6*) and *Aminopeptidase N* (*APN*) of rice yellow stem borer (YSB) on growth and development of insect. The bioassays involved injection of chemically synthesized 5′ FAM labeled 21-nt dsRNA into rice cut stems and allowing the larvae to feed on these stems which resulted in increased mortality and observed growth and development changes in larval length and weight compared with its untreated control at 12–15 days after treatment. These results were further supported by observing the reduction in transcripts expression of these genes in treated larvae. Fluorescence detection in treated larvae also proved that dsRNA was readily taken by larvae when fed on dsRNA treated stems. These results from the present study clearly show that YSB larvae fed on dsRNA designed from *Cytochrome P450* and *Aminopeptidase N* has detrimental effect on larval growth and development. These genes can be deployed to develop YSB resistance in rice using RNAi approach.

Keywords: yellow stem borer-YSB, double stranded RNA (dsRNA), growth and development, bioassays, cytochrome P450 (*CYP6*), aminopeptidase N (*APN*)

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INTRODUCTION

The rice yellow stem borer (YSB), *Scirpophaga incertulas* (Lepidoptera: Crambidae), is a widely distributed and destructive insect that causes significant yield losses to an extent of 10–90% by feeding on rice crop at all stages (Bandong and Litsinger, 2005). The YSB larvae that enter inside the stem cause death of the central shoot known as "dead hearts" at vegetative stage and unfilled panicles known as "white heads" at reproductive stage (Pathak and Khan, 1994). There are no resistant sources available for this deadly pest in cultivated rice germplasm (Brar and Khush, 1997). Although the option of expression of cry genes from *Bacillus thuringiensis* (Bt) is available, development of insect resistance in field has become one of the long standing issue with Bt food crops (Tabashnik et al., 2013). Given these circumstances, silencing of YSB genes through RNA

interference (RNAi) by inactivating the key insect genes leading to aberration in pest growth and metabolism (Yang et al., 2011) offers an alternative. RNAi is sequence-specific gene silencing at the post-transcription level, induced by double stranded RNA (dsRNA; Fire et al., 1998; Hamilton et al., 2002). Down regulation of the expression of specific target genes through RNAi has been widely used for genetic research in several insects (Price and Gatehouse, 2008). However, selection of crucial genes which have an important role in insect life cycle for a specific insect is still a challenge (Kola et al., 2015).

Cytochrome P450 monooxygenases (cytochrome P450s) are found in virtually all living organisms. It plays an important role in the metabolism of xenobiotics such as drugs, pesticides, and plant toxins (Scott, 2008). In insects, cytochrome P450s play a predominant role in the metabolism of insecticides, which often results in the development of insecticide resistance in insect populations (Zhou et al., 2010). Most insect cytochrome P450 genes belong to microsomal *CYP4, CYP6, CYP9, CYP28, CYP321,* and mitochondrial *CYP12* families which have been associated with detoxification processes allowing the insect to become tolerant or resistant to insecticides or host plant allelochemicals (Guo et al., 2011). Aminopeptidase N (*APN*) is a member of metallo enzymes present in larvae midgut of lepidopteran insects. It plays an important physiological role in dietary protein digestion (Marchler-Bauer et al., 2015). Inhibition of its activity in the midgut can result in detrimental effect on larval growth, development, and lead to larval mortality (Reed et al., 1999). *APNs* are mainly studied for their role as receptors in Cry toxin-induced pathogenesis in insects (Bravo et al., 2007). Expression of *APN*s was found in midgut and malphigian tubules (Wang et al., 2005). RNAi mediated silencing of *CYP6AE14* (Cytochrome P450 derivative) and *APN* in other lepidopteran insects like *Helicoverpa armigera*, *Plutella xylostella, Achaea janata***,** and *Spodoptera litura* resulted in significant down-regulation of corresponding transcript and protein expression causing larval growth arrest and mortality and development of lethal larval-pupal intermediates (Rajagopal et al., 2002; Mao et al., 2007; Sivakumar et al., 2007; Bautista et al., 2009; Ningshen et al., 2013).

The present work was aimed to examine the effect of dsRNA molecules on silencing of YSB genes *CYP6* and *APN* by feeding larvae on dsRNA treated cut stems. The dsRNAs fed larvae were examined to see the effect of silencing of these genes on insect growth and metabolism. Our bioassays simulated the *in vivo* mechanism of gene silencing and showed that dsRNA molecules can be taken up through the normal dietary path of YSB. Further, the target gene expression level was examined in control and dsRNA fed larvae which suggested silencing of the corresponding gene of YSB which is the first RNAi approach for YSB.

MATERIALS AND METHODS

Design of Double Stranded RNA and Prediction of Off Targets

To clone cDNAs of *CYP6* and *APN* genes, a total of 48 corresponding sequences of *CYP6* gene were downloaded from lepidopteron database, NCBI. Out of these, 10 sequences could find near homologies which were used for designing three primers. Similarly, to clone *APN*, total 47 sequences were downloaded from lepidopteron database, NCBI. Out of these, 25 sequences showed near homologies which were used to design two sets of primers from the conserved region through online tool (http://primer3.ut.ee/). Using these primers 750 bp cDNA of *CYP6* and 728 bp cDNA for *APN* were cloned from YSB and submitted to *Genbank* (*CYP6*: KC904274, *APN*: KF290773). These YSB cDNA sequences were used for identification of siRNA hotspots and dsRNAs were designed based on the Reynold rules (Reynolds et al., 2004). The designed dsRNA for *CYP6* sense strand was 5′-AGUUGAGAAUGAAAUGACUGA-3′ and the antisense was 3′-UCAACUCUUACUUUACUGACU-5′ which had high Reynolds score of six out of eight. The similar strategy was followed for designing dsRNA from *APN* gene, the designed dsRNA sense strand sequence was 5′ GACGACGUAUACUUAACUACU-3′ and the antisense was 3′-CUGCUGCAUAUGAAUUGAUGA-5′ which had the high Reynolds score eight out of eight. Care was taken for designed dsRNA not to have any unintended off targets, which was apparent by doing BLAST search. The designed dsRNAs were also tested in *in silico* to ascertain the fulfillment of different parameters for maximum silencing by using siMAX siRNA design tool (https://www.eurofinsgenomics.eu/en/dna-rna-oligonucleotides/custom-rna-oligos/simax-sirna.aspx). The selected dsRNA were chemically synthesized at Eurofins Genomics GmbH, Germany by attaching FAM (5′carboxy fluorescein) at 5′ position (**Figure 1A**).

Bio Assay

Adult moths of YSB were collected from the rice field at Indian Institute of Rice Research (IIRR), and released on susceptible *cv.* TN1 plants in glasshouse for egg laying. These egg masses were collected and transferred into plastic vials for hatching. The neonate larvae that hatched from a single egg mass were released on to ∼8 cm long TN1 stems and reared at 25 ± 2°C for 3 days (Padmakumari et al., 2013). For *in vitro* cut stem bioassay, fresh TN1cut stems (∼8 cm) were placed in petri plate with moistened filter paper. For standardization of optimum concentration of dsRNA required for assay, three different concentrations i.e., 10, 20, and 30 pM of dsRNA were chosen. The dsRNA was injected into cut stems using small insulin syringe with volume of 30 µl per stem. Buffer (1×) of dsRNAs was used as control. The cut stems were kept at room temperature for 1 h for dsRNA to spread completely within the stem. On each treated cut stem, three larvae of 3 day old were released with the help of a soft paint brush. The insects were reared at 25 ± 2°C, 65%RH and 16:8 h (L: D).The optimized 30 pM concentration of dsRNA was used to determine its effect on larva, initially the experiment was carried out with different dsRNA concentration *viz.,* 10, 20, 30, and 40 pM. (Figures showing the effect of these concentrations on YSB were submitted as Supplementary Material). We found that the response was good at 30 pM. Hence in all the experiments we used 30 µl of 30 pM dsRNA per 8 cm cut stem to evaluate the effect on the target insect. Each bioassay was carried with 15 stems in three replication ($N = 45$) and each experiment repeated twice to obtain uniform results. Various parameters like

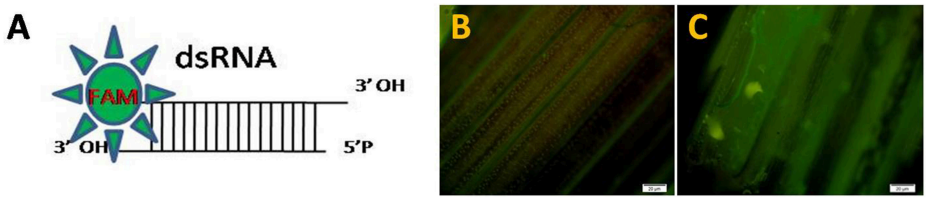

FIGURE 1 | (A) Schematic representation of dsRNA labeled with 5 FAM, Fluorescence detection of dsRNA in TN1 cut stems under microscope **(B)** Untreated **(C)** Treated cut stem.

insect molting, abnormal morphological changes in the larval growth and length, and survival were observed at 6, 12, 15 days after treatment (DAT). At each time interval, three samples (nine larvae) were selected for observation by opening stems through destructive sampling. The dead larvae were dissected to observe under fluorescent microscope (Olympus CH40, Japan).

Effects of dsRNA on YSB Larval Growth and Development

Larval Mortality

The mortality of larvae was recorded by opening the cut stems under a microscope at 3 day intervals before changing on to fresh treated stems. The number of surviving larvae per concentration of dsRNA was recorded at different time intervals of 6, 12, and 15 days after treatment. The mortality of the larvae in both treated and untreated stems were recorded.

Larval Weight and Length

Reduction in larval weight was obtained by calculating differences in the mean of larval weight in dsRNA treated samples with that of untreated control. Larval weight was measured in milligrams using an electronic balance and larval size with centimeter scale.

Fluorescent Detection of dsRNA

To confirm the dsRNA uptake by larvae which were fed on treated stems, fluorescence detection was conducted using a fluorescent microscope (Olympus CH40, Japan) in the range of 460–480 nm. To detect the dsRNA in whole larva as well as in the larval midgut, florescence was observed at 6, 12, and 15 days after treatment.

Statistical Analysis

Data on larval length, larval weight and percentage of larval mortality for *CYP6* and *APN* were analyzed by factorial ANOVA using Statistix 8.1 (Analytical Software, 2005). We observed that variance of mortality among the time intervals was slightly unequal, to remove this effect, percent mortality was arc sine transformed, this transformation was made following rules mentioned in Gomez and Gomez (1984).

Determination of Silencing

To detect the silencing of *CYP6* and *APN* in treated larvae, total RNA was extracted using Trizol reagent from 4 mg of single larvae. cDNA was synthesized according to manufacturer's instructions by using Prime Script™ cDNA Synthesis Kit (Takara, Japan) from the total RNA extracted at various time intervals. The Quantitative Real time PCR (qRT-PCR) reactions were carried out with SYBR Premix Ex TaqTM (Takara Bio INC, Japan) following the manufacturer's protocol using a Real time PCR LC-96 (Roche LightCycler® 96). The qRT-PCR primers were designed using the online tool Primer3 (http://primer3.ut.ee/). The primers used were *CYP6* Forward-GATTTTCGA CGTTACCCTCG Reverse- CCGCTGGGTTGGTAATTCC and *APN* Forward- AGGATTCAAGAGCTGGTCGT Reverse- GAT GACTTCGGTGTGAGGCA. For internal control, endogenous genes 18s rRNA from the Lepidopteron database and β-Actin were used (Kumar et al., 2009). Standard qRT-PCR procedures were followed with annealing of 60°C for 30 s. The specificity of the PCR reactions was monitored with melting curve analysis and gel electrophoresis. The relative gene expression data were analyzed by LC96 qPCR and by using the $2^{-\Delta\Delta Ct}$ method as described previously (Schmittgen and Livak, 2008).

Effect of dsRNA on Pink Stem Borer (PSB)

To check the specificity of RNAi effect and off-target effect of dsRNA, the designed dsRNAs were tested on rice pink stem borer (*Sesamia inferens*) through *in vitro* bioassay. Bioassays were performed as like YSB and observation were taken on larval length and weight. The data was analyzed statistically by *T*-test using Statistix 8.1 software (Analytical Software, 2005).

RESULTS

Cloning of cDNAs from YSB and Designing dsRNA

In this study, systematic efforts were made to demonstrate the silencing of *CYP6* and *APN* genes in YSB through *in vitro* studies using rice cut stems injected with corresponding dsRNAs. cDNAs of *CYP6* and *APN* were cloned, *CYP6* cDNA of YSB showed 70% identity with *Chilo suppressalis*, 69% with *Spodoptera*, 64% with *Helicoverpa,* and only 69% with Rice Leaf roller *Cnaphalocrocis medinalis*. Similarly, *APN* of YSB showed 71% identity with *Chilo suppressalis*, 89% with *Spodoptera*, 82% with *Helicoverpa,* and very less 69% with Rice Leaf roller *Cnaphalocrocis medinalis*. Among the siRNA hotspot regions, the region which does not have any homology to the related species was selected to design 21-nucleotide dsRNA. The designed two dsRNA sequences did not match to the any of the related species as well with mammalian species available in Genbank which indicates that it does not have the off targets. The cut stem assay was designed

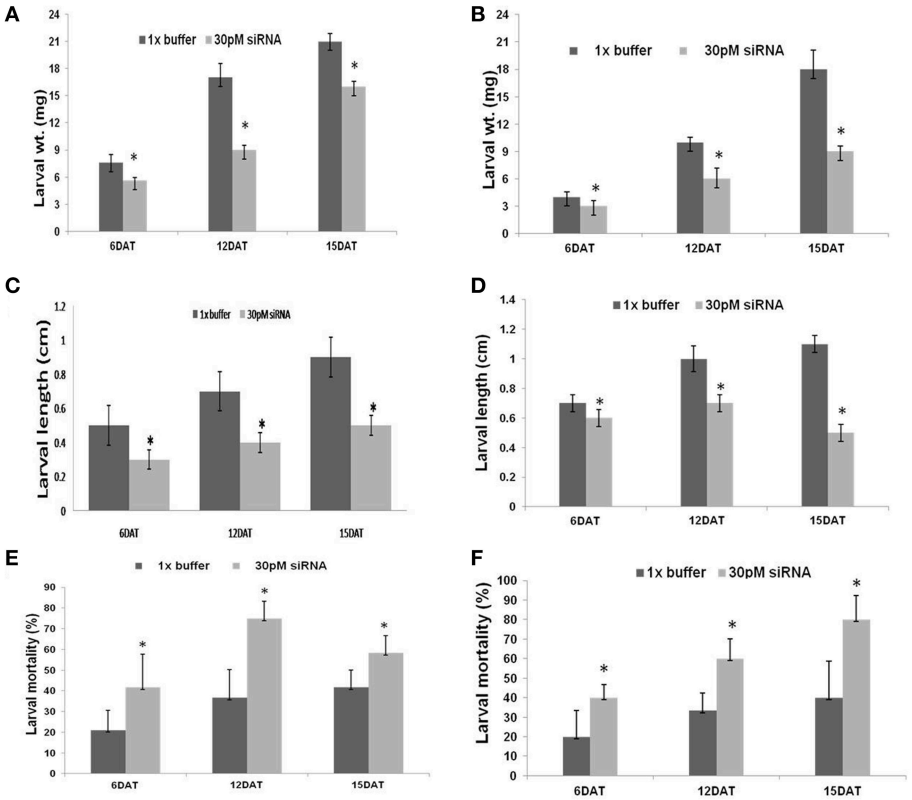

FIGURE 2 | (A) Effect of *CYP6* 30 pM dsRNA on YSB larval weight at different time intervals, *Indicates that larval weight in treatments were significantly different ($N = 3$, $F = 60.27$, DF = 11, $P < 0.001$) from larval weight in control. DAT -days after treatment. **(B)** Effect of *APN* 30 pM dsRNA on YSB larval weight at different time intervals, *Indicates that larval weight in treatments were significantly different ($N = 3$, $F = 28$, DF = 12, $P < 0.002$) from control, DAT- days after treatment. **(C)** Effect of *CYP6* 30 pM dsRNA on YSB larval length at different time intervals, *Indicates that larval lengths in treatments were significantly different from control larval length. **(D)** Effect of *APN* 30 pM dsRNA on YSB larval length at different time intervals, *Indicates that larval length were significantly different ($N = 3$, $F = 35.64$, DF = 14, $P < 0.0001$) from larval length in control. **(E)** Effect of *CYP6* 30 pM dsRNA on YSB larval mortality at different time intervals, *Indicates that larval mortality in treatments were significantly different ($N = 4$, $F = 9.13$, DF = 18, $P < 0.007$) from control larval mortality. **(F)** Effect of *Amino* 30 pM dsRNA on YSB larval mortality at different time intervals,*Indicates that larval mortality were significantly different ($N = 5$, $F = 12.73$, DF = 24, $P < 0.0016$) from control larval mortality.

so as to simulate a real time situation wherein the larva bore the stem pieces and enter into stem. As YSB is a monophagous pest of paddy, we used cut stems of TN1 (highly susceptible variety) for standardization of dsRNA concentration. We found 30 pM concentration of dsRNA could effectively silence the *CYP6* and *APN* genes and showed reduced growth and development, whereas, 10 and 20 pM concentration of dsRNA showed very less effect on larval growth (Supplementary Figure 1).

Effect of dsRNA on Growth and Development

The dsRNA of *CYP6* and *APN* had a pronounced effect on growth and development of the larvae. Larvae fed with dsRNA specific to the *CYP6* and *APN* showed significant inhibition of growth in a time dependent manner. Treated larvae (dsRNA) showed significant reduction in growth and development characters like larval length and weight compared with untreated larvae at regular time intervals. Larvae fed on *CYP6* dsRNA showed maximum weight reduction (8 mg) at 12 DAT and minimum was at 6 DAT as compared to its untreated control (**Figure 2A**). dsRNA designed from *APN* also showed significant larval weight and length reduction compared to its untreated larvae. The larval weight reduction was maximum at 15 DAT (**Figure 2B**). There was a significant reduction in larval length with increase in exposure to dsRNAs. Treated larvae showed a mean reduction in length of 0.45 cm at 15 DAT, 0.3 cm at 12 DAT, and 0.25 cm at 6 DAT compared to untreated control larvae (**Figures 2C,D**).

Effect of dsRNA on Larval Mortality

Mortality of treated larvae in both dsRNAs increased from 6 to 15 DAT. The highest mortality rate of 74.95% was observed at 12 DAT in *CYP6* (**Figure 2E**) and 80% at 15 DAT in *APN* treatment (**Figure 2F**; Supplementary Tables 1A,B). Florescent microscope observation before and after the release of larvae in the treated and untreated stems confirms that the dsRNA abundance and translocation within the injected stem (**Figures 1B,C**; Supplementary Figure 2). *CYP6* dsRNA fed larvae showed the presence of fluorescence in larval midgut at 6, 12, 15 DAT and it increased with the time course which was

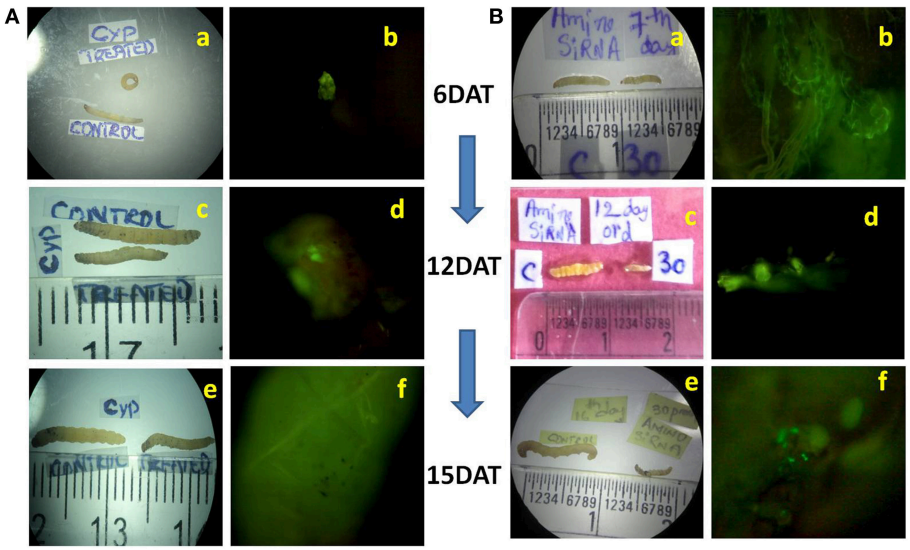

FIGURE 3 | (A) Effect of *CYP6* dsRNA on YSB larvae fed on treated TN1 cut stems, Growth reduction in treated larvae at **(a)** 6th DAT, **(c)** 12th DAT **(e)** 15th DAT after feeding on dsRNA treated TN1 cut stems. Presence of fluorescence in larval midgut indicating that dsRNA was ingested by larvae when fed on treated stems at **(b)** 6th DAT, **(d)** 12th DAT, **(f)** 15th DAT. **(B)** Effect of *Amino* dsRNA on YSB larvae fed on treated TN1 cut stems. Growth reduction in treated larvae at **(a)** 6th DAT, **(c)** 12th DAT **(e)** 15th DAT after feeding on dsRNA treated TN1 cut stems. Presence of fluorescence in larval midgut at **(b)** 6th DAT, **(d)** 12th DAT, **(f)** 15th DAT.

also proportional to the increase in larval mortality as well as decrease in larval weight (**Figure 3A**). Similarly, we found that fluorescence in larval midgut in *APN* dsRNA fed larvae was proportional to the effect on larval growth (**Figure 3B**). These observations indicate that the designed dsRNA were systemic in nature, and through oral feeding could reach the targets genes, bind to them, thus affecting the larval growth and development.

Confirmation of Gene Silencing by qRT-PCR

We have observed the reduction in the expression level of *CYP6*, *APN* in all the stages of dsRNA treated samples, whereas, maximum silencing was observed at 12 DAT in *CYP6*, and 15 DAT in case of *APN*. Quantitative RT–PCR revealed, *CYP6* expression in treated larvae was reduced by 3.5, 3.0 fold in 12, 15 DAT, respectively. In case of *APN,* the fold change in gene expression was increased by reducing its expression 2.9, 5.6, 13.5 fold in 6, 12, and 15 DAT compared to the control larvae (**Figures 4A,B**). The reduction in transcript levels in treated samples correlated with bioassay data for *CYP6* and *APN* genes. So these results demonstrated that both genes have key roles in maintaining YSB growth and development.

Effect of dsRNA on PSB

The PSB larvae were reared on the rice TN1 cut stems where YSB specific dsRNAs was injected into the stems. Interestingly, no abnormalities were observed in PSB larvae when fed on treated stems of both dsRNA. In *APN* dsRNA treatment, the PSB larvae lengths were similar to that of untreated control at 6, 12, and 15 DAT. Larval weight (mg) was also similar to the control at 6 and 12 DAT. However, decrease in larval weight was observed at 15 DAT (30 $\pm$ 1.0 mg), which is significantly lower (*P*-value 0.0055) compared to untreated control (49.0 $\pm$ 1.0 mg) but larval mortality was not observed. Whereas, in case of *CYP6* dsRNA, we did not observe any changes in larval length and weight between control and treatments at three different time intervals. (Supplementary Table 2). Interestingly, larvae were quite active in both the treatments and mortality was not at all observed (Supplementary Data Videos 1–3) and pupae were formed. This clearly indicates that, the designed dsRNAs are specific to the YSB and they did not show any abnormal effect on PSB (**Figure 5**).

DISCUSSION

The success of RNAi for insect control depends on identification of suitable genes for RNAi. The candidate gene should not only have insecticidal effects on the target pests, but also safe toward other organisms, natural enemies and human beings (Agarwal et al., 2012; Kola et al., 2015). The present study showed that RNAi technology is an effective strategy for silencing the key genes involved in the metabolic activities of YSB. RNAi-triggered gene suppression through uptake of dsRNAs has been reported for many insect species belonging to Coleoptera, Diptera, Hemiptera, Hymenoptera, Isoptera, Lepidoptera, and Orthoptera (Huvenne and Smagghe, 2010).

In this study, two important genes *viz.*, *CYP6* and *APN* were selected based on their earlier successful reports of silencing in lepidopteron pests (Rajagopal et al., 2002; Mao et al., 2007; Sivakumar et al., 2007; Bautista et al., 2009; Crava et al., 2010; Yang et al., 2010; Zhang et al., 2011; Edi et al., 2014).The Cytochrome P450 monooxygenases are present in almost all organisms including insects. They play vital roles in hormone

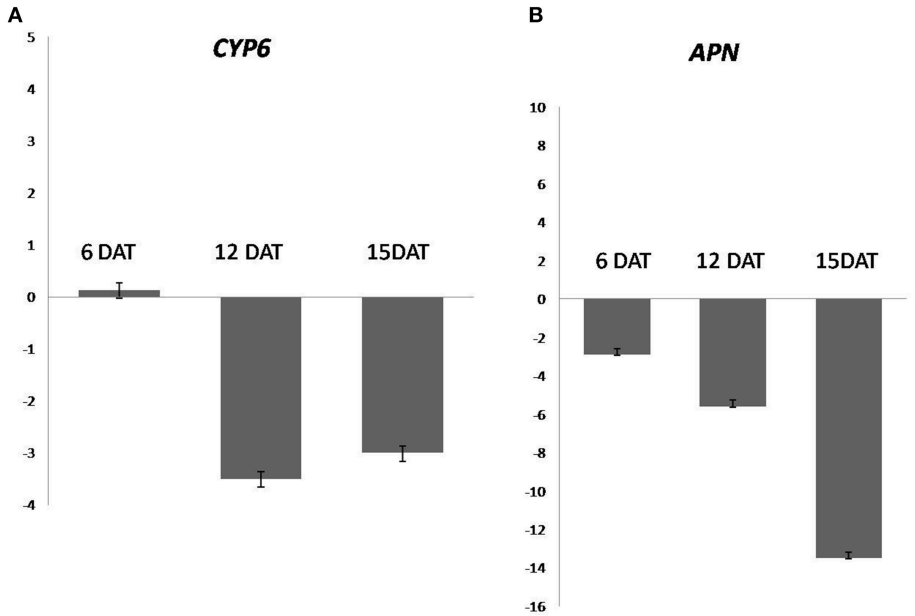

FIGURE 4 | Relative expression fed on TN1 cut stems treated with *CYP6*-dsRNA and *APN*-dsRNA in an individual bioassays. β-actin and 18 s were used as an internal controls **(A)** expression of *CYP6* gene from larvae sampled at 6, 12, 15 days after treatment along with respective controls. **(B)** Expression of *APN* in 6, 12, 15 days after treatment along with respective controls.

regulation, metabolism of xenobiotics and in biosynthesis or inactivation of endogenous compounds (Kola et al., 2015; Yu et al., 2015). Similarly, Aminopeptidase N (*APN*s) is primarily involved in dietary protein digestion majorly located at the midgut epithelium. *APNs* are known to cleave a single amino acid residue from the N-terminus of oligopeptides, preferentially the neutral amino acids (Wang et al., 2005; Pigott and Ellar, 2007). As, these two genes are known to play very important role in insect growth and development (Mao et al., 2007; Ningshen et al., 2013), we selected them as a candidate for silencing in YSB. Wang et al. (2015) succeeded in silencing *CYP6AB14* by injecting dsRNA derived from *CYP6AB14* into *S. litura* and found reduced transcript levels of *CYP6AB14* and increased developmental abnormalities and higher mortality rates. Zhang et al. (2013), also observed larval mortality and reduced growth in *H. armigera* upon larval feeding on dsRNA designed from *CYP6B6*. Similar results were also reported with transgenic expression of *CYP6AE14* in tobacco and cotton (Hodgson et al., 1995; Mao et al., 2007, 2011). Recently, Jin et al. (2015) designed dsRNAs targeted to *Chitin synthase* (*Chi*), *CYP6AE14,* and *V-ATPase* genes and expressed in the tobacco chloroplasts for control of *Helicoverpa* and observed reduced transcription of target genes in the insect midgut and stunted larval growth. Silencing of *APN1* was reported in Castor Semilooper, *A. janata* (Ningshen et al., 2013).

For successful design of dsRNAs, there is a need to identify the specific regions or motif of key pest genes. To attain this, for the first time partial length of two candidate genes, *CYP6* and *APN* were cloned from the YSB through PCR based strategy. Though YSB belongs to lepidopteron family, *CYP6*

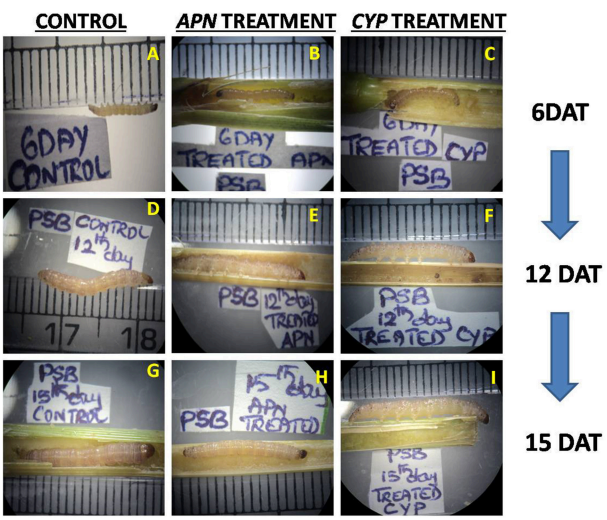

FIGURE 5 | PSB larvae fed on rice TN1 cut stems, 6th DAT **(A)** Control, **(B)** *APN* treated, **(C)** *CYP* treated. 12th DAT **(D)** Control, **(E)** *APN* treated, **(F)** *CYP* treated. 15th DAT **(G)** Control, **(H)** *APN* treated, **(I)** *CYP* treated.

showed 69% identity and *APN* 82% identities with *Helicoverpa* indicating significant differences. The unique regions of two YSB genes were selected for designing dsRNA, following Reynolds rules (Reynolds et al., 2004). The higher scores with these principles suggest having more stability and greater silence effects (Horn et al., 2010). Much of the commercial software currently available for designing dsRNAs follow Reynolds rule, Ui-Tei

rule, Amarzguioui rule, and Tuschl rule (Naito and Ui-Tei, 2012). The dsRNAs designed in this study of *CYP6* and *APN* genes had the highest score based on Reynolds rule, which might have positive influence of their higher expression. There were some reports of using long dsRNA for effective RNAi than small dsRNA for insects (Miller et al., 2012; Li et al., 2015), however, recent reports indicated the more effectiveness of small dsRNAs insect control (Kumar et al., 2009; Naito and Ui-Tei, 2012; Gong et al., 2013). Our designed dsRNA from *CYP6* lies in the principle domain of CYP450 group of enzymes, which belongs to haem-thiolate proteins involved in the oxidative degradation of various compounds. This class of proteins has domains viz., haem-binding loop (with an absolutely conserved cysteine that serves as the 5th ligand for the haem iron), the proton-transfer groove and the absolutely conserved EXXR motif in helix K forms a principle domain. Similarly *APN (*zinc-dependent metallopeptidases) is a Type II integral membrane protease belongs to Gluzincin family (thermolysin-like peptidases or TLPs), this family consists of several zinc-dependent metallopeptidases including M1 peptidases and know to consist of a small N-terminal cytoplasmic domain, a single transmembrane domain and a large extracellular ectodomain that contains the active site. The dsRNA designed is lies in the part of active site of this enzyme so we expect that it binds to the target effectively.

Generally insect bioassays have been carried out through introducing dsRNA into an organism by microinjection (Ghanima et al., 2007), soaking and oral feeding through artificial diet (Mao et al., 2007; Chen et al., 2008). Since, YSB is a monophagous pest and does not have artificial diet; we resorted to rearing on rice cut stems, so as to simulate a real time situation where larva bore the stem pieces and enter into stem. In earlier studies cut stem assay were used to assess the toxicity of various toxins and transgenics against YSB (Nayak et al., 1997; Nguyen Huu Ho et al., 2001; Ramesh et al., 2004; Padmakumari et al., 2013). We found lower concentration of dsRNA (30 pM) was effective for silencing of target genes. Kumar et al. (2009) also found that 50 nM concentrations were effective for silencing Acetyl cholinesterase *(Ache)* gene in *H. armigera.* Similarly, Bautista et al. (2009) reported that 250 ng dsRNA targeting *CYP6BG1* delivery through droplet feeding to 4th instar larvae of *Helicoverpa* greatly reduced the *CYP6BG1* expression. To track accurately the presence of dsRNA in various insect tissues, FAM was used to label the dsRNA and confirmed their systemicity in insect tissue. Earlier reports have indicated the use of fluorescent dyes like Fluoroscein isothiocyanate (FITC), Cyanine Cy-3, Cy-5, and FAM to track the movement and binding of dsRNA in the tissues (Urwin et al., 2002; Karim et al., 2010; Hui et al., 2011; Wuriyanghan et al., 2011; Bolognesi et al., 2012; Li et al., 2015).

Our results indicated the increase of YSB larval mortality rates in feeding assay with dsRNA of *CYP6* and *APN* genes. Change in growth and developmental characters were observed from very early larval developmental stages by showing reduced larval growth (larval weight and length), delayed molting and led to death. Interestingly, none of the larvae entered into pupae form. We observed an increase in fluorescence signal with increase in time of exposure which indicates the signal amplification. These observations were correlated with qRT-PCR experiments data.

The designed dsRNA sequences did not match with mammals or related species which are present in GenBank and beneficial insects which suggests that the designed dsRNA molecules may be specific to target insect and may not have unintended effects. Further to make sure of specificity of these molecules, bioassays were carried out in PSB, another stem borer of rice. The results indicated that dsRNAs designed from *CYP6* and *APN* did not showed significant effect on larval length and weight except in case of 15 DAT with *APN* treatment. Further investigations are required to fully understand the delayed response of *APN* treatment in PSB larval weight. There was no morality in both the cases, larvae were quite active completed their instars and entered in to the pupal stage. These results suggest that the YSB dsRNAs of *CYP6* and *APN* genes could not find targets in PSB which further confirms that the designed dsRNAs were more specific to YSB.

In our study, we conclusively proved the key functions of Cytochrome P450 and Aminopeptidase N enzymes in various metabolic pathways of YSB such as normal growth and also its involvement in many stages of development. The present results strongly suggest that these *CYP6* and *APN* genes can be potential targets for insect-control, and insect-resistant transgenic plants may be obtained through RNAi-mediated silencing of insect genes. The off targeting study through *in silico* and *in vivo* showed that the designed dsRNA from these genes are highly specific to insects and do not have any unintended effects on other organisms.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiment: MSM, SKM, KVSR, PR, APP, SMB, and VRB.

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Winged Pea Aphids Can Modify Phototaxis in Different Development Stages to Assist Their Host Distribution

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The pea aphid, *Acyrthosiphon pisum* (Harris) (Hemiptera: Aphididae), shows wing polyphenism (winged and wingless morphs) in its life cycle. The winged morph is adapted for dispersal; its two developmental adult stages (for dispersal and reproduction) are based on its breeding periods. The two morphs show different phototactic behavior and the winged can change its preference to light according to the developmental stages. To determine the mechanism and ecological functions of phototaxis for *A. pisum*, we first investigated the phototaxis of the two aphid morphs at different stages and analyzed the phototactic response to lights of different wavelengths; the correlation between alate fecundity and their phototactic behaviors were then studied. Finally, we focused on the possible functions of phototaxis in aphid host location and distribution in combination with gravitaxis behaviors. Negative phototaxis was found for breeding winged adults but all the other stages of both winged and wingless morphs showed positive phototaxis. The reactions of the aphids to different wavelengths were also different. Nymph production in winged adults showed negative correlation to phototaxis. The dopamine pathway was possibly involved in these behavior modifications. We speculated that winged adults can use light for dispersal in the early dispersal stage and for position holding in the breeding stage. Based on our results, we assume that light signals are important for aphid dispersal and distribution, and are also essential for the pea aphids to cope with environmental changes.

Keywords: phototaxis, distribution, dopamine, insect behavior, *Acyrthosiphon pisum*

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INTRODUCTION

The pea aphid *Acyrthosiphon pisum* (Harris), exhibits wing phenotypes at various stages of its life cycle (Braendle et al., 2006). Normally, winged morph is adapted for dispersal and wingless morph for reproduction. The winged and wingless phenotypes in aphids differ in morphology, physiology, and behavior. The winged morph exhibits an elaborate sensory system for flight and host plant location, such as more fully developed compound eyes, ocelli, and longer antennae with more rhinaria as compared with the wingless morph. The wingless morph lacks wings and the wing musculature for dispersal, but it has a faster development time and a larger body size for production than the winged morph (Braendle et al., 2006; van Emden and Harrington, 2007; Brisson, 2010). Density (tactile stimulation) and nutrition (host plant quality) are considered to

be the key environmental cues affecting the transformation from winged to wingless morphs. This represents an adaptation of the pea aphid to the environment (Johnson, 1965; Lees, 1967; Sutherland, 1969; Sutherland and Mittler, 1971; Wratten, 1977).

Taxis is the movement of an organism in response to different stimuli including physical, chemical, and biological ones; movements of an organism toward or away from a stimulus, are defined as positive or negative taxis, respectively. Many types of taxis have been identified, such as chemotaxis (by chemicals), electrotaxis (by electric current), gravitaxis (by gravity), hydrotaxis (by moisture), phototaxis (by light), and thermotaxis (by temperature) (Hader, 1987; Mori and Ohshima, 1995; Alon et al., 1999; Pringault and Garcia-Pichel, 2004; Rezai et al., 2010). Phototaxis is a locomotory movement toward or away from a light stimulus for an organism. Many organisms show phototaxis from prokaryotes to eukaryotes including plants and animals (Bulkowski and Meade, 1983; Armitage and Hellingwerf, 2003; Chen et al., 2012). Insects also display such behaviors; for instance, moths, wasps, and whiteflies (Summers, 1997; Castrejon and Rojas, 2010; Chen et al., 2012; Yang et al., 2012). Phototaxis has been widely used in pest control; for instance, in the invention of light traps and yellow card traps, which are based on insect phototaxis to ultraviolet and yellow light (Bowden, 1982). On the other hand, the phototaxis of an organism may change during its life time. It has been recorded that some insect species can change their phototaxis or geotaxis in certain situations, such as developmental stages and starvation (De Ruiter and van der Horn, 1957; Barrett and Chiang, 1967; Ben-Shahar et al., 2003; Gong et al., 2010). Phototactic changes in particular situations may also be occurring in some other animals, such as fish, nudibranch, and stomatopod species (Dingle, 1969; Bulkowski and Meade, 1983; Miller and Hadfield, 1986).

Phototactic behaviors are stimulated by light, and light in different wavelengths may affect phototaxis in insects in different ways. Many insects prefer blue light or ultraviolet, and others can be attracted by green or yellow light. Insects can react to more than one wavelength bands (Coombe, 1981; Yang et al., 2003; Mazza et al., 2010; Yamaguchi et al., 2010). Light with different wavelengths can stimulate visual organs and lead to different reactions in insects (Yokoyama, 2000; Briscoe and Chittka, 2001). Studies show sensors in both ocelli and compound eyes could be functional in phototactic behaviors (Garrey, 1918; Gilbert, 1994; Lazzari et al., 1998). The study of one aphid species *Megoura viciae* labeled some photoperiodic photoreceptors including red-light sensitive photoreceptor-specific proteins (CERN-956) and long-wavelength sensitively photoreceptor-specific proteins (COS-1) in ventral neuropile of protocerebrum and eyes (Gao et al., 1999).

As a complex behavior in insects, phototaxis is controlled by the insect neural system. Previous studies in insects and other animal species showed neurotransmitters (dopamine, serotonin, and so on) played specific roles in phototactic behavior. Dopamine (3, 4-dihydroxyphenethylamine, DA) is an important neurotransmitter and hormone of the catecholamine and phenethylamine families (Joh and Hwang, 1987). Dopamine has been well studied in human and other mammals. Studies reveal that dopamine plays important roles in motor function, reward, learning, aggression, addiction, memory, and some other behaviors in invertebrates, as well as those in vertebrates (Coleman and Neckameyer, 2004; Rauschenbach et al., 2012; Martin and Krantz, 2014). Dopamine is also a key chemical in cuticle maturation (sclerotization and melanization) (Gallot et al., 2010). Researches in *Drosophila* showed that dopamine functioned in phototaxis, while dopamine-deficient flies were also defective in positive phototaxis (Neckameyer et al., 2001; Riemensperger et al., 2011). Serotonin played a key role in phototactic behavior in the honeybee, *Apis mellifera* (Thamm et al., 2010). There is also some evidence in other animals: dopamine could prolong while serotonin could repress positive phototaxis in *Bugula neritina* (Pires and Woollacott, 1997), and serotonin has been reported as negatively modifying phototaxis in a species of crab *Carcinus maenas* (McPhee and Wilkens, 1989).

An understanding of phototaxis would be helpful in studies of aphid dispersal and distribution. Previous studies in many aphid species showed that aphids display phototactic behaviors and normally show a positive preference (Kennedy et al., 1961; Kennedy and Booth, 1963; Hajong and Varman, 2002). During our many years of pea aphid rearing, we found that different aphid morphs did have different phototactic behaviors. The understanding of this changeable phototactic behavior and its underlying mechanism in *A. pisum* reflects the adaptation of *A. pisum* to its ecological conditions.

Gravitaxis response might affect aphid's movement patterns and response for upward climbing on host plants. Only few gravitaxis related studies could be found in *Drosophila melanogaster* (Toma et al., 2002; Armstrong et al., 2006) and there is no gravitaxis receptor researches about aphid to our data. Some studies mentioned aphids exhibited negative gravitaxis (Brunissen et al., 2010; Le Roux et al., 2010) or positive and negative gravitaxis (Pettersson et al., 2007) without further detailed studies. The gravitactic behavior of the pea aphid was then determined.

To determine the underling mechanisms of phototactic behaviors, we first recorded the phototactic response of two morphs of the pea aphid in different nymphal instars to white light. Based on the different phototactic results in winged adults, we analyzed the relationship between phototactic response and fecundity; and then we focused on the phototactic behaviors of selected aphids to different wavelengths of light. We also designed experiments to analyze the possible connections between neurotransmitters (dopamine, octopamine, and serotonin) and aphid phototactic behavior changes. Finally, combining with the results for gravitaxis of the pea aphid, we designed an experiment to study the possible functions of phototaxis in pea aphid host-distribution.

MATERIALS AND METHODS

Aphids and Plants

A red morph of pea aphid was collected from Lanzhou, Gansu Province, China, and reared on broad bean (*Vicia faba* L., var. "Jinnong") under a long-day condition (16L: 8D; 20 ± 1°C)

for more than 30 generations at the Key Laboratory of Applied Entomology, Northwest A&F University, Yangling, Shaanxi, China. All wingless aphids were reared at a low density (<30 aphids per 4-week-old plant) for more than three generations before they were used. A high density (30 aphids per 2-weeks old plant seedling) was used to stimulate wing formation. Selected winged aphids were reared at a low density (<30 aphids per 4-weeks old seedling) before they were used in all subsequent experiments.

Phototaxis Behaviors in Different Instars of Winged and Wingless Pea Aphids

Phototactic preference of the aphids were determined by comparing the numbers of aphids that moved to a lighted area or remained in a dark area as shown in **Figure 5A**. The arena was made of a transparent plastic petri dish (90 mm in diameter). A lighted area was formed by placing a piece of white filter paper (45 mm in diameter) in the bottom for light reflection; a cold light (KL 1500 LCD, Zeiss, German, set color temperature at 6500 K, 2000 lx) was used as a light source. The lens was focused on the filter paper forming a sharp-edged circle (45 mm in diameter, the same size as the filter paper). The remaining area remained in the dark. The lighted area was one fourth and the dark area was three fourth of the arena. We tested the phototactic preference of wingless nymphs (first and second instars), winged nymphs (third and fourth instars), wingless adults (1-day old), and winged adults (newly emerged and 8-days old, **Figure 1D**). All aphids were starved for 6 h before they were used. The experiments were undertaken in a dark room at 10:00 a.m., and each treatment lasted for 30 min. During the experiment, 50 starved aphids were placed in each arena, and numbers of aphids moved to the lighted area and those remained in the dark area were counted. Each experiment was repeated 15 times.

Correlation between Nymphs Production and Phototaxis in the Winged Adults

Newly emerged winged pea aphid adults were reared at a low density (<30 individuals per seedling). The nymphs produced were individually counted every day for 8 days. Phototaxis was also determined for 8 days as described above. The correlation of nymphs produced and phototaxis was analyzed for each treatment. Each experiment was repeated 15 times.

Phototaxis of Starved Winged Adults

Eight-days old winged adults in their reproduction period were starved for 6, 8, 10, 12, 14, 16, or 18 h before they were used in the phototaxis experiments as described above. We selected wingless adults (3 days old), and winged adults (1-day old and 8-days old) in the experiments. Each treatment was repeated 15 times.

Phototactic Behaviors to Different Light Wavelengths

In this experiment, we determined the effects of three light wavelengths on phototaxis of different morphs of the pea aphid. The three wavelengths (red: >600 nm; green: 400–600 nm; blue: 350–500 nm) were obtained by using band filters on the lenses of a cold light source (KL 1500 LCD) (**Figure 5B**). Phototaxis of the aphids was determined as described above. Each treatment was repeated 15 times.

Transcription in *DDC*, *TβH*, and *TPH*

Detection of rate-limiting enzyme transcription levels in neurotransmitter production was used for neurotransmitter analysis. Considering the high L-DOPA contents in the host plants *V. faba* (Ingle, 2003; Zhang et al., 2016), we picked *DDC* (DOPA Decarboxylase) downstream for dopamine analysis; *TβH* (Tyramine β-Hydroxylase, converted L-tyramine to octopamine) was selected for octopamine and *TPH* (Tryptophan Hydroxylase, and converted L-tryptophan to 5-Hydroxy-L-tryptophan) for serotonin (Figure S2).

To analyze expression differences in *DDC*, *TβH*, and *TPH*, wingless and winged adults in the first and eighth day were snap-frozen and dissected between the T1 and T2 segments (**Figure 2F**). The head and T1 segment (to avoid affecting embryos in the abdomen) were used for transcription tests, and 15 individuals were prepared for one repetition. There experiments were repeated three times. Aphid samples were frozen using liquid nitrogen immediately after collection. RNA was extracted with RNAiso Plus (Takara, Japan), and cDNA was synthesized using a PrimeScript™ RT reagent kit with gDNA Eraser (Takara, Japan). Quantitative real-time PCR (qRT-PCR) was performed with SYBR® Premix Ex Taq™ II (Takara, Japan) in an IQ-5 system (Bio-Rad, Berkeley, California, USA). The primers were designed by Primer-BLAST of NCBI online (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) (Table S1).

Dopamine Antagonist Treatments

To further analyze the dopamine functions in phototactic behavior, dopamine receptor antagonist was injected to modify the dopamine level in the pea aphids. Based on the aphid's locomotion and pretests, 1-day old winged adults were used for the experiments. SCH23390 (R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2, 3, 4, 5-tetrahydro-1H-3-benzazepine hydrochloride, CAS 125941-87-9, sigma, D1 receptor antagonist) (Peczely et al., 2014) and Sulpiride ((−)-5-(aminosulfonyl)-N-[(1-ethyl-2-pyrrodinyl)methyl]-2-methoxy-benzamide, CAS 15676-16-1, sigma, D1 and D2 receptor antagonist) (Hauber and Lutz, 1999) were used in the experiments. A high dose (2.5 mM) of antagonist was used for injection as described by Vergoz et al. (2007).

The methods of injection of dopamine antagonist solution were described by Barron et al. (2007) and Scheiner et al. (2002). A glass needle (P-97 Micropipette Puller, Sutter, CA, USA; a pulling program: Pull = 100, VEL = 100, and Time = 100) attached to the Nanoject II™ Auto-Nanoliter Injector (Drummond Scientific Company, USA) was used in the injection. Dopamine antagonist solution was injected into the thorax (T3 segment) of the aphids, and thoracic injections were made through the fissure at the base of their hind legs (**Figure 2F**); 200 nl solution was injected per aphid; ddH_2O in the same dose (200 nl) was used as a control. The aphids were then moved to *V. faba* after treatment. All samples were used for phototaxis tests after 8 h host rearing followed by 6 h starvation. Phototaxis

experimental protocol are described above. Injected and control winged aphids (1-day) for *DDC* were used for transcription analysis. The transcription analysis protocol was followed as described above.

Gravitaxis Analysis

Bottom Release

Fifty aphids were put on the bottom dish of the device and sealed by aluminum foil. The cylinders were divided into five parts by height (A, B, C, D, and E). The experiments lasted for 3 h, and the aluminum foil was then removed, and number of aphids in each position was counted.

Top Release

Fifty selected aphids were put into the container. The dish with aphids was covered for 1 h; the device was sealed with aluminum foil immediately after aphid release. The experiment lasted for 3 h, and the aluminum foil was then removed, and number of aphids in each position was counted. The gravitaxis of the pea aphids was determined as shown in **Figure 5C**.

The Effect of Light on Aphid Spatial Distribution on Host Plants

This experiment was conducted to determine possible effects of lighting direction on phototactic behavior and spatial distribution of pea aphids on host plants (**Figure 5D**). Four-week-old *V. faba* were used as the host plants. A piece of filter paper made into a cone was used to hold aphids. A cold light source (KL 1500 LCD, Zeiss, German, set color temperature at 6500 K, 8000 lx) was placed on top or bottom of the plant depending on experiment design. A no-light treatment was used as a control. Fifty aphids were used each time. The leaves on the plant were marked from top to bottom as A, B, C, and D (**Figure 5D**), and number of aphids on each leaf was counted 3 h later. The experiment was conducted in a dark room, and each experiment was repeated 10 times.

Statistical Analysis

All experimental data generated were collected and subjected to statistical analysis using Student's *t*-test, and one-way ANOVA; means were separated using Duncan test; Kolmogorov–Smirnov test were used for distribution analysis and Pearson correlation test were used for correlation analysis (SPSS version 22; SPSS Inc., Chicago, IL, USA).

RESULTS

Phototactic Behavior Analysis in Different Instars of Winged and Wingless Pea Aphid

The phototaxis responses in the winged adults showed significant difference. Based on the counts of aphids in the dark and light areas, the aphids were strongly attracted to the light except to the 8-days old winged adults which showed negative phototaxis ($t = -3.108$, $df = 28$, $P = 0.006$; **Figure 1A**). The 1-day-old winged adult showed the strongest taxis to the light while the first and second instars showed weaker light preference than other positive-phototactic aphids ($F = 51.682$, $df = 8, 126$, $P < 0.0001$, **Figure 1A**).

Correlation Analysis between Nymphs Production and Phototaxis in Winged Adult Pea Aphid

The newly emerged winged adults in the first 2 days after emergence showed significant positive phototaxis (1 d, $t = -7.502$, $df = 17$, $P < 0.0001$; 2 d, $t = -6.014$, $df = 17$, $P < 0.0001$, **Figure 1B**); and showed slight negative phototaxis in the following 4 days (3 d, $t = -0.301$, $df = 17$, $P = 0.767$; 4 d, $t = 2.720$, $df = 17$, $P = 0.015$, 5 d, $t = 1.542$, $df = 28$, $P = 0.141$; 6 d, $t = 1.003$, $df = 17$, $P = 0.330$, **Figure 1B**); obvious negative phototaxis were observed at the seventh and eighth days (7 d, $t = 2.851$, $df = 17$, $P = 0.011$; 8 d, $t = 4.698$, $df = 17$, $P < 0.0001$, **Figure 1B**). There was an increase in nymph production during this process ($F = 73.579$, $df = 7, 232$, $P < 0.0001$, **Figure 1B**), which was negatively correlated with phototaxis (Pearson $r = -0.724$, $P = 0.042$).

Changes in Phototaxis of Winged Adults (Breeding Period) in Starvation

Eight-days old winged adults could develop positive phototaxis when staved. This happened after 12 h of starvation (6 h: $t = 4.698$, $df = 17$, $P < 0.0001$; 8 h: $t = 2.337$, $df = 17$, $P = 0.032$: 10 h: $t = -0.025$, $df = 17$, $P = 0.981$: **Figure 1C**), which could change into significant positive phototaxis (12 h: $t = -3.171$, $df = 17$, $P = 0.006$; 14 h: $t = -3.924$, $df = 17$, $P = 0.001$; 16 h: $t = -6.747$, $df = 18$, $P < 0.0001$; 18 h: $t = -8.098$, $df = 17$, $P < 0.0001$: **Figure 1C**).

Phototactic Behavior Analysis in Response to Different Light Wavelengths

The aphids showed different phototactic reactions to different light wavelengths. In the red light treatment (>600 nm), wingless adults showed significant positive phototaxis ($t = -2.676$, $df = 17$, $P = 0.016$), and 1-day old winged adults showed stronger preference for red light ($t = -9.816$, $df = 17$, $P < 0.0001$). Eight-days old winged adults showed no reaction to red light ($t = -0.253$, $df = 17$, $P = 0.803$; **Figure 1E**). In the green light treatment (400–600 nm), wingless adults also showed significant positive phototaxis ($t = -6.718$, $df = 17$, $P < 0.0001$); 1-day-old winged adults showed no reaction to green light ($t = -0.695$, $df = 17$, $P = 0.496$); while 8-days old winged adults showed significant negative phototaxis ($t = 2.505$, $df = 17$, $P = 0.023$; **Figure 1F**). In the blue light (350–500 nm) experiment, both wingless adults and 1-day old winged adults showed significant positive phototaxis (wingless adults: $t = -20.270$, $df = 17$, $P < 0.0001$; winged adults: $t = -18.947$, $df = 17$, $P < 0.0001$), and for 8-days old winged adults the difference was not significant ($t = 1.559$, $df = 17$, $P = 0.137$; **Figure 1G**).

Dopamine, Octopamine, and Serotonin Analysis

By analyzing rate-limiting enzyme transcription levels of dopamine (*DDC*), we found that 8-day winged adults showed

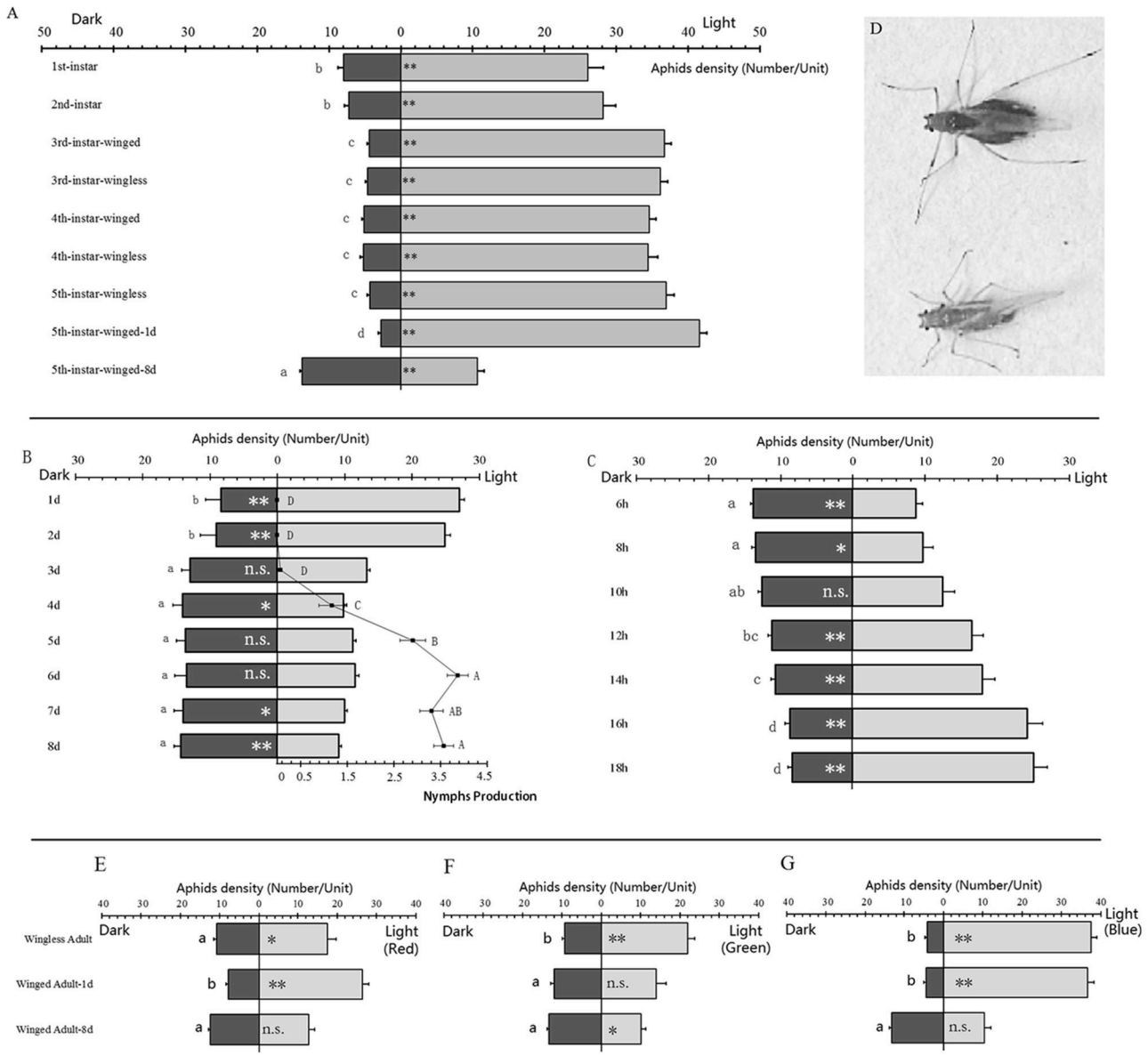

FIGURE 1 | Phototactic response in different stages and wing forms of *Acyrthosiphon pisum.* The phototaxis results in different stages of *A. pisum* **(A)**. The fecundity and phototaxis correlations in winged adults during their development **(B)**, and the phototaxis changing in starvation of winged adults in breeding period **(C)**; winged adult in pre-breeding (left) and breeding (right) period **(D)**. Phototaxis in wingless adults 1-day and 8 d winged adults under light with different wavelengths, in red (>600 nm, **E**), green (400–600 nm, **F**) and blue (350–500 nm, **G**). Data were based on aphids' density (number/unit). The light area has 1 unit while dark area has 3 units. * and ** indicate significant different at $P < 0.05$ and $P < 0.01$, respectively (Student's *t*-test). The different letters next to the bars indicate significant differences at $P < 0.05$ (Duncan test).

more significant down-regulation than the others ($F = 7.635$, $df = 2, 6$, $P = 0.022$; **Figure 2A**). In octopamine ($T\beta H$) analysis, wingless adults showed the lowest expression level among all aphid stages and 1-day-old winged adults showed the highest ($F = 8.385$, $df = 2, 12$, $P = 0.005$; **Figure 2B**). The serotonin analysis (*TPH*) for wingless adults showed more significant down-regulation than for another two aphids ($F = 8.385$, $df = 2, 9$, $P = 0.010$; **Figure 2C**).

Dopamine Antagonists Treatment

After 1-day-old aphid adults were injected, their phototactic behaviors also changed. All injected aphids showed no response to light (SCH23390, $t = -1.539$, $df = 28$, $P = 0.135$; sulpiride, $t = -0.319$, $df = 28$, $P = 0.752$; H_2O, $t = -1.296$, $df = 28$, $P = 0.206$; **Figure 2E**).

By analyzing *DDC* transcription level between injected aphids and the control, we found that *DDC* showed significant

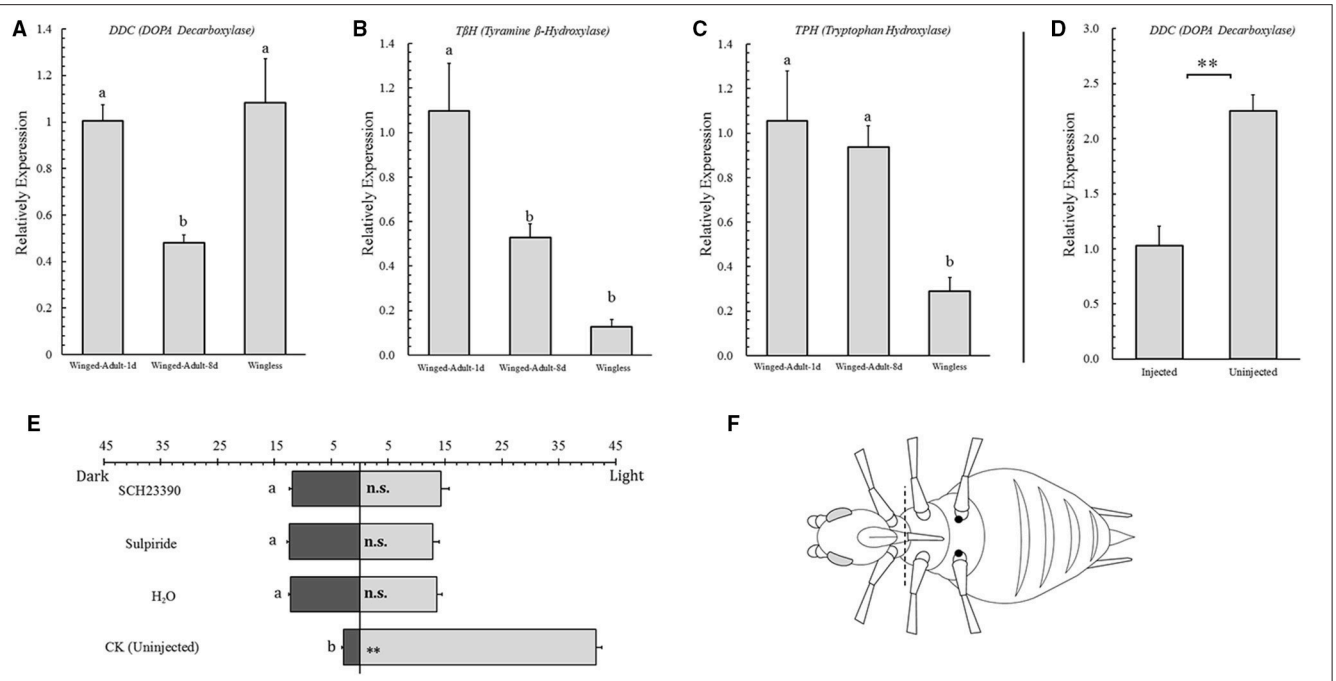

FIGURE 2 | Relative expression analysis of key enzymes of neurotransmitters in selected *Acyrthosiphon pisum* and phototactic tests after injection experiments in *A. pisum*. Relative expression levels of *DDC* (DOPA Decarboxylase, **A**), *TβH* (Tyramine β-Hydroxylase, **B**) and *TPH* (Tryptophan Hydroxylase, **C**) in three selected *Acyrthosiphon pisum* (1-day and 8-day winged adults and wingless adults) and their phototaxis tests **(E)**; relative expression levels of *DDC* between injected and uninjected *A. pisum* **(D)**. Dissection (dotted line) and injection (dark spots) position are showing in **(F)**. Each value represents the mean ± SEM from independent determinations. ** in both **D** and **E** indicate that the means are significantly different at $P < 0.01$ (Student's *t*-test), and different letters on top of the bars indicate significant differences at $P < 0.05$ (Duncan test).

down-regulation in injected individuals ($t = -5.334$, $df = 4$, $P < 0.006$; **Figure 2D**).

Gravitaxis Analysis

Gravitaxis analysis indicated that no obvious gravitaxis in all aphids tested. In the bottom release experiment, all aphids were still at the bottom 3 h after the experiment (1-day-old winged adults, $F = 357.987$, $df = 4, 70$, $P < 0.0001$; 8-days-old winged adults, $F = 105.846$, $df = 4, 70$, $P < 0.0001$; wingless adults, $F = 213.327$, $df = 4, 70$, $P < 0.0001$; **Figures 3A,C,E**). In the top release experiment, most aphids were still at the top, although some moved downward. One-day-old winged adults showed a wider spread (1-day-old winged adults: $F = 19.088$, $df = 4, 70$, $P < 0.0001$; 8-days-old winged adults: $F = 46.870$, $df = 4, 70$, $P < 0.0001$; wingless adults: $F = 19.649$, $df = 4, 70$, $P < 0.0001$; **Figures 3B,D,F**), which represented some degree of positive gravitaxis.

Light Attraction Analysis in Pea Aphid Host Spatial Distribution

The spatial distribution of 1-day old winged adults was affected by lighting direction, and more aphids aggregated at the lighting source (A: $F = 8.339$, $df = 2, 27$, $P = 0.001$; B: $F = 57.709$, $df = 2, 27$, $P < 0.0001$; C: $F = 3.065$, $df = 2, 27$, $P = 0.063$; D: $F = 42.685$, $df = 2, 27$, $P < 0.0001$). More than one third of the aphids (bottom lighting: 52.67%; top lighting: 37.67%; no lighting: 56.33%) were not on the host after the experiments, and the bottom-lighting attracted more aphids than the top-lighting and no-lighting treatments ($F = 49.242$, $df = 2, 27$, $P < 0.0001$; **Figure 4A**).

For 8-day winged adults, the distribution was also affected by lighting direction (Figure S1A), and only position A (marked on **Figure 5D**) had a significant increase in aphids numbers in the bottom-lighting treatment (A: $F = 37.175$, $df = 2, 27$, $P < 0.0001$; **Figure 4B**). No differences were obtained in other positions (B: $F = 8.157$, $df = 2, 27$, $P = 0.002$; C: $F = 1.287$, $df = 2, 27$, $P = 0.293$; D: $F = 11.532$, $df = 2, 27$, $P < 0.0001$; **Figure 4B**) between the top- and bottom-lighting treatments. However, the aphid distribution was different in the no-light treatment. Less than 3% of aphids were not on plant in the two lighting treatments (bottom lighting: 3%; top lighting: 1%), while 42% failed to find their host without light; the difference was significant ($F = 6.592$, $df = 2, 27$, $P = 0.005$; **Figure 4B**).

The distribution of wingless adults on hosts was also strongly affected by light (Figures S1A,C). Numbers of aphids were different in positions B and D (marked on **Figure 5D**); more aphids aggregated at the lighting source (A: $F = 18.118$, $df = 2, 27$, $P < 0.0001$; B: $F = 12.427$, $df = 2, 27$, $P < 0.0001$; C: $F = 1.586$, $df = 2, 27$, $P = 0.223$; D: $F = 1.917$, $df = 2, 27$, $P = 0.167$; **Figure 4C**). Nearly 29% aphids did not find their host without light, and most or all aphids (bottom lighting: 5%; top

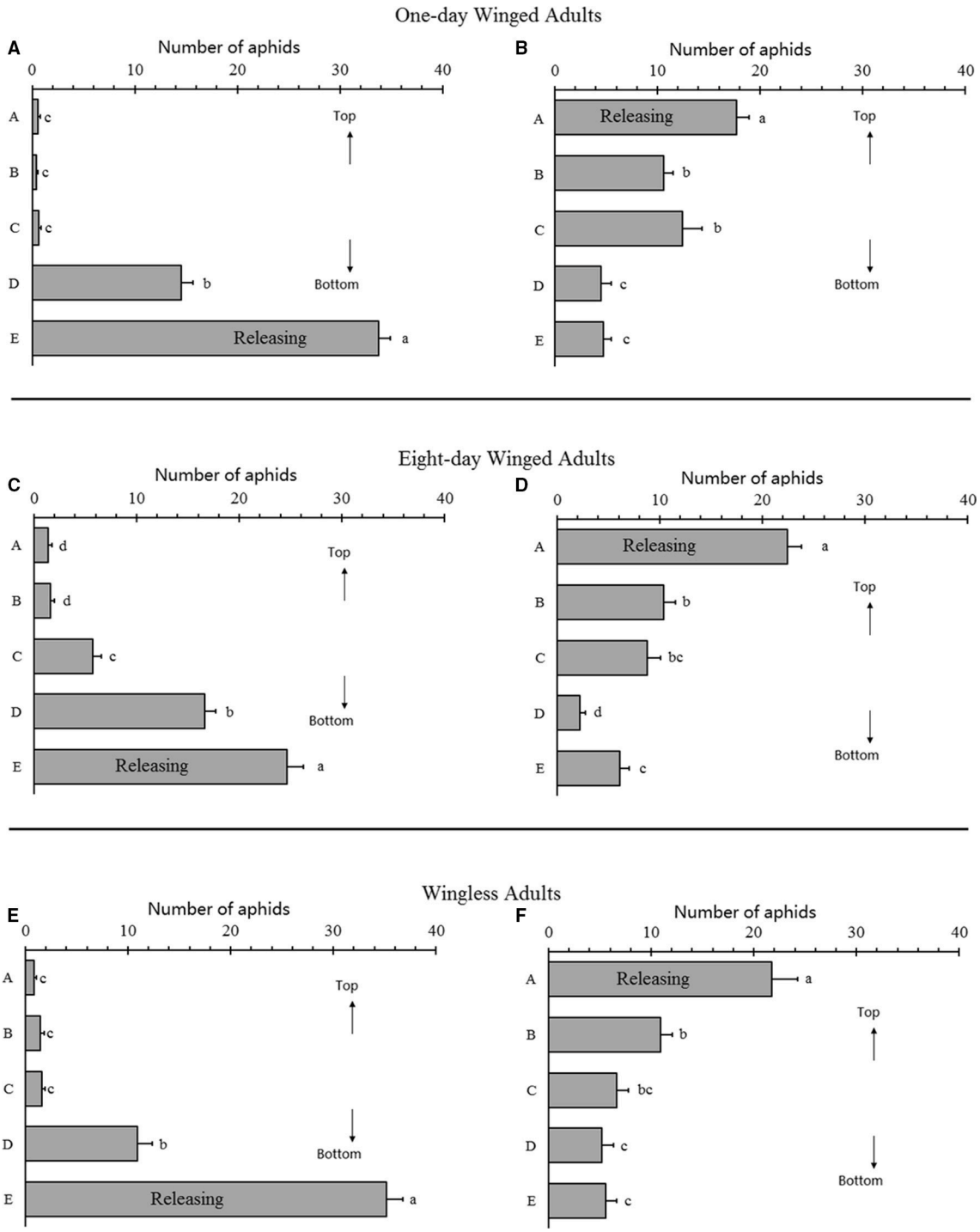

FIGURE 3 | Gravitaxis analysis of selected *Acyrthosiphon pisum*. One-day winged adults in bottom-releasing **(A)** and top-releasing **(B)**; 8-day winged adults in bottom-releasing **(C)** and top-releasing **(D)**, wingless adults in bottom-releasing **(E)** and top-releasing **(F)**. Different letters next to the bars are significantly different ($P < 0.05$, Duncan test).

lighting: 0%) moved to the plant in the top lighting treatment ($F = 76.649$, $df = 2, 27$, $P < 0.0001$; **Figure 4C**). Numbers of aphids unable to find their hosts in the no lighting treatments for all treatments. More wingless aphids found their host plants than the winged adults without light ($F = 12.296$, $df = 2, 27$, $P < 0.0001$; **Figure 4D**).

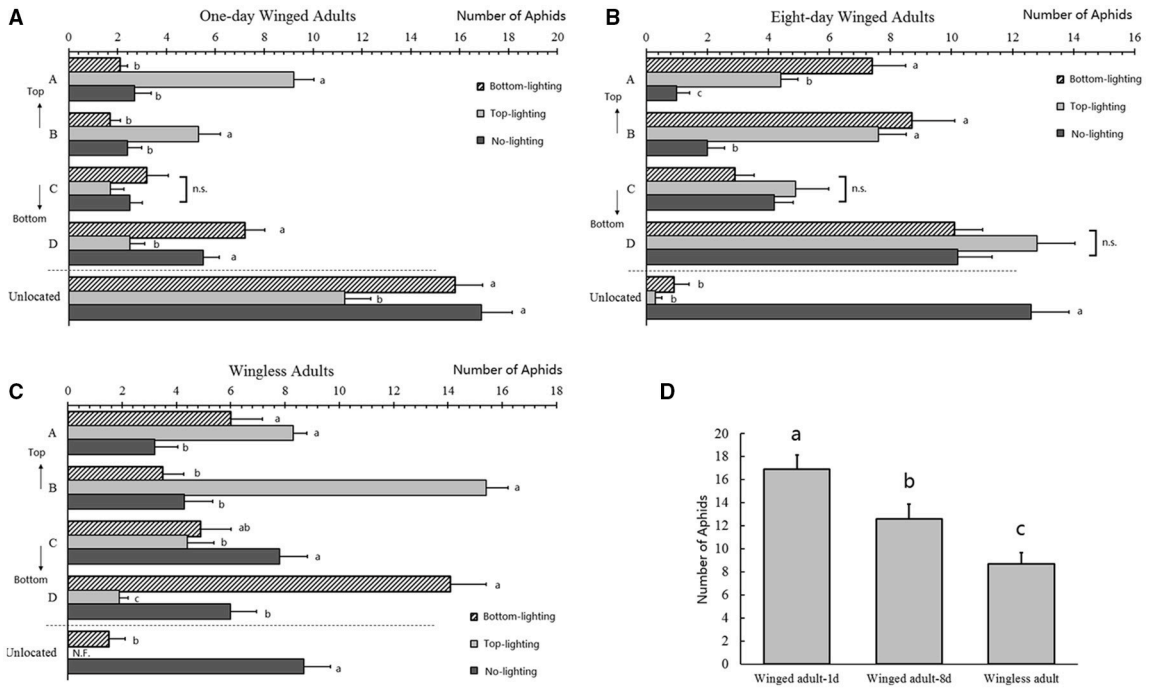

FIGURE 4 | Distributions of *Acyrthosiphon pisum* under different lights. Distributions of 1-day old winged adults **(A)**, 8-days old winged adults **(B)**, and wingless adults **(C)** as indicated by the aphids numbers in each part under different lights. Aphid lost in the three selected aphids were shown in **(D)**. Different letters next to the bars are significantly different ($P < 0.05$, Duncan test).

DISCUSSION

Our experiments showed that winged *A. pisum* could actually change their phototaxis during their development, and this change can assist aphids in dispersal and host distribution. We found that wingless pea adults showed positive phototaxis while winged adults could change phototaxis from strongly positive to negative depending on their breeding situation. Adults in their reproduction period could regain their phototaxis to positive under starvation. Different morphs showed different reactions to different light wavelengths. We speculated that the dopamine modification pathway was related to phototactic behaviors. These behaviors might assist the aphid to optimize its distribution on host plants

Insects can react to some physical stimuli for environmental adaptation. Phototaxis is one of these behaviors that can be stimulated by light signals (Bowden, 1982; Miller and Hadfield, 1986; Summers, 1997; Briscoe and Chittka, 2001). We found that the pea aphid could also show phototaxis (Figure S1B). The wingless and winged nymphs and wingless adults showed only positive phototaxis (**Figure 1A**). These results were similar to the studies of Hajong and Varman (2002) on *Sitobion rosaeiformis*. But we also detected that winged adults could change their phototaxis, and these changes were related to their fecundity; the more nymphs they laid the stronger preference for dark they show (**Figure 1B**). This special behavior appears unique to the winged *A. pisum* adults.

Dopamine and serotonin pathways have been reported in phototaxis modification (McPhee and Wilkens, 1989; Pires and Woollacott, 1997; Neckameyer et al., 2001; Riemensperger et al., 2011). In our investigation, we found that dopamine might be altering phototaxis. Only 8-day-old winged adults showed down-regulation in *DDC,* and they also showed slight negative phototaxis. Expression levels of *DDC* in 1-day-old winged adults and wingless adults were relatively higher than 8-day-old winged adults, and both of them represented positive phototaxis (**Figures 1A**, **2A**). It exhibited a connection between *DDC* expression and phototaxis. This provided similar results to those in *D. melanogaster* and *B. neritina* which high dopamine can increase positive phototaxis (Pires and Woollacott, 1997; Neckameyer et al., 2001; Riemensperger et al., 2011). We assumed that dopamine pathway would function similarly in *A. pisum* as well, and the dopamine pathway downstream (dopamine receptors and dopamine re-uptaking) also need to be studied. In the meantime, we assumed that the octopamine (based on *TβH* expression) might represent the locomotion abilities in the pea aphids (dispersal winged adult > breeding winged adult > wingless adult), which dispersal winged morphs showed higher regulation than the other two morphs we selected. Serotonin (based on *TPH* expression) exhibited differences between the wingless aphid and the two winged forms (**Figures 2B,C**), and the mechanism behind needs to be investigated. Previous studies reported that serotonin could also modify phototaxis (McPhee and Wilkens, 1989; Thamm et al., 2010), but in our experiments we observed no connection between serotonin and phototaxis in the pea aphids (**Figures 1A**, **2C**).

We attempted to reveal the dopamine receptors functions in phototaxis by using dopamine antagonist. The treatment was

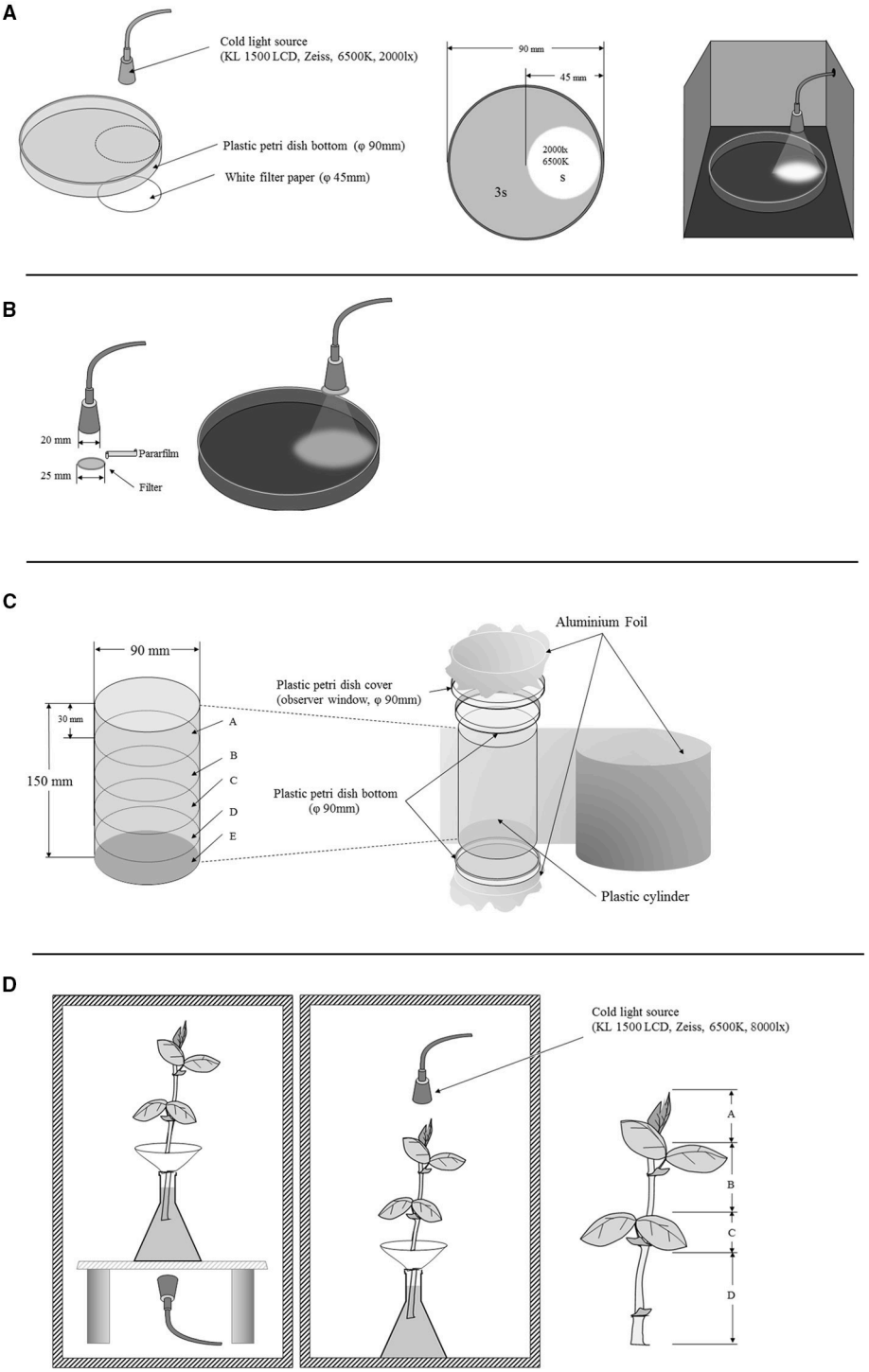

FIGURE 5 | The set-up of the phototaxis experiment of *Acyrthosiphon pisum*: for phototactic response in different stages and wing forms of *Acyrthosiphon pisum* (A); for phototactic response with different wavelengths (B); for gravitaxis of *A. pisum* (C); and for distributions under different lights (D).

relatively unsuccessful and no differences were observed in our experiment. But to our surprise, all treated aphids showed a weak response to light. We have noticed that injection (physical injury) could affect dopamine production (*DDC* expression). It could lead to declining dopamine levels in all injected aphids (aphids feed on the fluid in plant phloem. Due to the difficulty to feed the

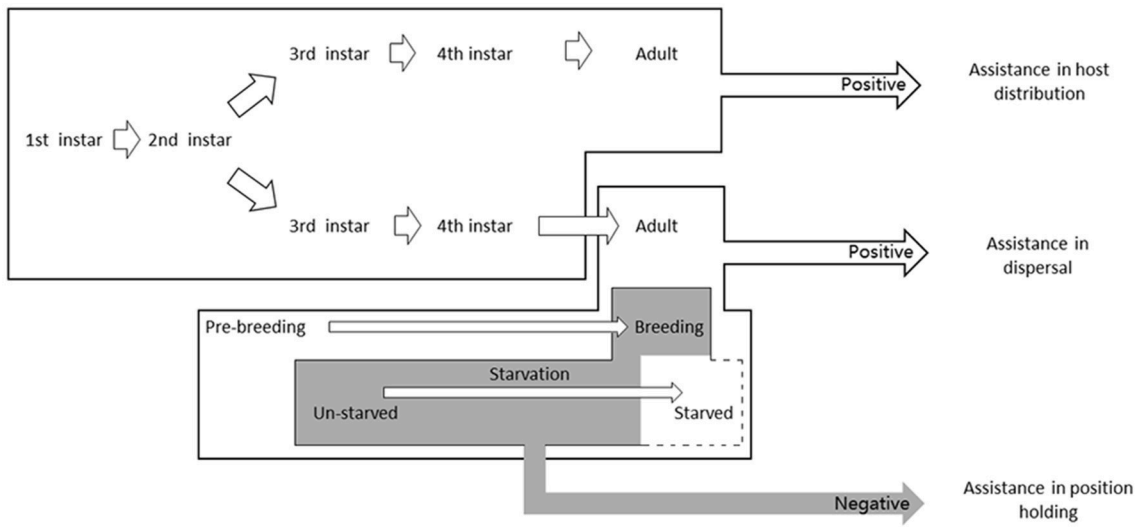

FIGURE 6 | The hypothesis of phototaxis functions in different *Acyrthosiphon pisum* morphs. Winged aphids can change their phototactic responses, which help them to disperse during their pre-breeding period and to prevent them to fly away from the host plants during their reproduction period; for the wingless morphs, their positive phototaxis could assist them in locating best parts of host plants.

aphids dopamine antagonists, our results showed that artificial diet (poor nutrition) could also affect dopamine pathway) (**Figure 2D**). Both of our *DDC* expression detection of injected and control aphids showed down-regulation, which means all the treated aphids had a decline in dopamine level. This result also supported our assumption regarding the function of dopamine in aphid phototaxis, and the weak negative phototaxis that observed might be caused by dopamine biosynthesis declining. But the multiple functions of dopamine revealed that phototactic behavior is not likely to be simple, and we still need a better way to obtain precise results.

Considering that the winged adult was the only morph that responded to population dispersal and its strong response to light, the changes in phototactic behaviors would affect their movement patterns in winged *A. pisum*. It is reasonable that the negative phototactic behavior of breeding winged aphids has a special ecological function that related to light signal, and aphids' movement patterns (host distribution) in host was considered as a breakthrough. The results in the spatial distribution of the aphids on plants indicated that positive phototaxis of the wingless aphids could reach plant top. If the light source changed downward, their distribution could be affected. Because the direction of sunlight is normally from above, we assumed that sunlight could be a beacon for aphids to identify where the host top is located and to move toward it. For winged aphids, we believe that the multiple functions of phototaxis could assist other behaviors. Combing with our studies, we hypothesized that in the early stage of winged adults (newly emerged, pre-breeding), strong positive phototaxis could help the aphids reach the top of a plant and fly away. After they found a host and started to develop into breeding winged morphs, positive phototaxis would be unwise, and the aphids need to find a way to keep themselves sedentary. Therefore, based on our results, we assumed that an altering phototaxis could help in solving this problem. On the other hand, if the host was not familiar to the aphids in wild condition, negative phototaxis could help them to walk downward and get away from the immature part (buds and new leaves), which probably have a stronger resistance to aphids or being predated by nature enemies. Starvation normally represents a lack of nutrition, and long duration of starvation could reverse phototaxis, which may lead aphids back to the top position in order to fly away in our opinion. Changeable phototaxis is common in other animals, representing a flexible adaptation to the environment (De Ruiter and van der Horn, 1957; Barrett and Chiang, 1967; Dingle, 1969; Bulkowski and Meade, 1983; Miller and Hadfield, 1986; Ben-Shahar et al., 2003; Gong et al., 2010). Our researches revealed that *A. pisum* had a similar physiological character. The hypothesis of its ecological functions need more evidences.

Previous studies by Hajong and Varman (2002) in *S. rosaeiformis* also indicated the involvement of phototaxis in aphid distribution, but this aphid species could reach the host bud without light. We found surprisingly that our tested *A. pisum* aphids displayed a strong decline in host finding abilities without lighting, and compared with the two winged individuals, wingless aphids showed better host finding ability (**Figure 4**).

All aphid in the experiment slightly displayed positive gravitaxis (**Figure 3**). These results showed the importance of light (or vision) in host location for pea aphid. It has been discussed in earlier studies, vision of the insects may be even more important than olfaction in host finding (Reeves et al., 2009; Reeves, 2011; Machial et al., 2012), and our work also supports this finding.

In conclusion, different pea aphid morphs could display different phototaxis to light stimuli, and light in different wavelength bands could also give different stimuli to aphids.

We noticed that light in short wavelengths produced strong positive or negative effects on aphids, and light in middle or long wavelength bands had weaker or no effects on them. The tested aphid morphs showed different reactions to specific light wavelengths. Based on previous studies of aphid's vision organs, different eye constructions (compound eyes, and ocelli missing) might be responsible for the differences in reaction to light (Braendle et al., 2006; Brisson, 2010). Dopamine, a neurotransmitter, might play a certain rule between light signal detection and behavioral response in pea aphid.

Winged pea aphids can changes their phototactic responses, which help them to disperse during their pre-breeding period and to prevent them to fly away from the host plants during the period of their reproduction. For the wingless morphs, their positive phototaxis could assist them in locating hosts (**Figure 6**). We found that the aphids were observed almost unable to locate their host without light so that light signals are extremely important for aphid dispersal and distribution, representing a significant adaptation to ecological environments.

AUTHOR CONTRIBUTIONS

YZ and TL designed the research; YZ and XW performed research; XJ and HT provided assistance; YZ, XW, and HT analyzed data; YZ and XW wrote the manuscript; XJ, HT, and TL edited the manuscript; and XW and YZ revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fphys.2016.00307

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Heterogeneity of the Peripheral Circadian Systems in *Drosophila melanogaster*: A Review

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Circadian rhythms in organisms are involved in many aspects of metabolism, physiology, and behavior. In many animals, these rhythms are produced by the circadian system consisting of a central clock located in the brain and peripheral clocks in various peripheral tissues. The oscillatory machinery and entrainment mechanism of peripheral clocks vary between different tissues and organs. The relationship between the central and peripheral clocks is also tissue-dependent. Here we review the heterogeneous nature of peripheral circadian clocks in the fruit fly *Drosophila melanogaster* and their dependence on the central clock, and discuss their significance in the temporal organization of physiology in peripheral tissues/organs.

Keywords: circadian rhythm, circadian clock, cryptochrome, *Drosophila*, peripheral oscillator, physiological rhythms, molecular oscillatory mechanism

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Circadian rhythms are often observed in animal behavior, physiology, and gene expression (Aguilar-Roblero et al., 2015). In the fruit fly, *Drosophila melanogaster*; *one* of the most useful model organisms for the study of the circadian system, the central clock consists of about 150 clock gene expressing neurons in the brain (Taghert and Shafer, 2006; Shafer and Yao, 2014). The central clock regulates behavioral rhythms including locomotor behavior and sleep-wake cycles and is clustered in two neural groups, lateral neurons (LNs) and dorsal neurons (DNs). LNs are subdivided into three clusters, small ventral LNs (s-LNv), large ventral LNs (l-LNv), and dorsal LNs (LNd). DNs are subdivided into three clusters, DN_1, DN_2, and DN_3(reviewed by Helfrich-Förster, 2005; Hermann-Luibl and Helfrich-Förster, 2015). The s-LN_v are necessary and sufficient for sustained locomotor rhythm in constant darkness (DD) (Helfrich-Förster, 1998; Renn et al., 1999) and are the most important cluster in the central circadian network (Grima et al., 2004; Stoleru et al., 2004, 2005). The dynamic neuronal network between the clock neurons is regulated by various neurotransmitters including pigment dispersing factor (PDF), which is expressed in the s- and l-LNvs (Shafer and Yao, 2014; Yao and Shafer, 2014; Hermann-Luibl and Helfrich-Förster, 2015) and fine-tunes the circadian rhythm to adapt to the environmental cycles (Miyasako et al., 2007; Yao and Shafer, 2014).

In addition to the central clock, peripheral clocks reside in various organs and tissues and likely regulate rhythms in organ/tissue specific functions (**Table 1**). Some peripheral clocks have been characterized only by immunohistochemistry to detect the oscillation of clock proteins or by reporter assays with luciferase expression driven by clock gene promotors (Giebultowicz and Hege, 1997; Plautz et al., 1997).Molecular studies revealed that the peripheral clocks are based on

Abbreviations: CLK, CLOCK; *Clk*, *Clock;* CNS, central nervous system; CRY, CRYPTOCHROME; *cwo, clockwork orange;* CYC, CYCLE; *cyc*, *cycle;* DD, constant darkness; DN, dorsal neurons; EAG, electroantennogram; LD, light dark; LN, lateral neurons; LNv, ventral lateral neurons; s-LNv, small lateral neurons; l-LNv, large lateral neurons; LNd, dorsal lateral neurons; LPN, lateral posterior neurons; MT, Malpighian tubules; *norpA, no receptor potential A;* PDF, pigment dispersing factor; PER, PERIOD; *per, period;* PG, prothoracic gland; RG, ring gland; TIM, TIMELESS; *tim*, *timeless.*

cell-autonomous molecular oscillation and that most directly respond to light when kept in culture conditions (Plautz et al., 1997). Further detailed studies focusing on molecular oscillations in the periphery and their output rhythms have revealed diversity in the circadian organization among the peripheral circadian systems (e.g., Myers et al., 2003; Ito et al., 2008; Krupp et al., 2013). In the present review, we summarize and discuss the features of peripheral oscillators, their heterogeneity, and their relationship to the central clock.

HETEROGENEITY IN MOLECULAR MACHINERY: THE FUNCTION OF CRY IN PERIPHERAL CLOCKS

The circadian oscillation in central clock neurons is based on transcriptional/translational feedback loops that involve the transcription factors CLOCK (CLK) and CYCLE (CYC) (Tataroglu and Emery, 2015). CLK and CYC dimerize and induce transcription of *period* (*per*) and *timeless* (*tim*) genes. The translated products, PER and TIM, form a heterodimer that suppresses the activity of CLK-CYC to produce rhythmic expression of *per* and *tim* with a period of about 24 h. Light input causes degradation of TIM via the blue light receptor, CRYPTOCHROME (CRY), causing the circadian clock to be reset (Stanewsky et al., 1998; Suri et al., 1998; Ceriani et al., 1999; Busza et al., 2004). In addition to CRY, the visual system, including compound eyes, ocelli, and Hofbauer-Buchner eyelets, are involved in photic entrainment of the central clock (Stanewsky et al., 1998; Helfrich-Förster et al., 2001).

The oscillatory mechanism is shared by the central and the peripheral oscillators (Hardin et al., 2003); however, the function of CRY varies between peripheral oscillators: two different roles for CRY in the periphery have been suggested. (1) CRY functions as a photoreceptor and a core clock component (Stanewsky et al., 1998; Ivanchenko et al., 2001; Collins et al., 2006), and (2) CRY serves only as a photoreceptor (Ito et al., 2008) (**Figure 1A**). The following two sections provide examples for tissues/organs within which CRY has different roles.

CRY Serves as both a Photoreceptor and a Clock Component

The dual roles of CRY as a photoreceptor and a clock component are well-demonstrated in Malpighian tubules (MT) (Stanewsky et al., 1998; Ivanchenko et al., 2001). In this organ, a light pulse given during the subjective night induces the degradation of TIM, resulting in a reset of the phase of circadian oscillation. Light-induced TIM degradation was eliminated in cry^b, hypomorphic mutants lacking functional CRY. In addition, the PER and TIM oscillations in the MT disappeared in cry^b mutants (Stanewsky et al., 1998; Ivanchenko et al., 2001). These studies suggest that CRY has roles both in light entrainment and in the molecular oscillatory machinery of the clock. Another example is the antenna. Its response to odorants, as measured by an electroantennogram (EAG), increases at night and decreases during the day under light-dark (LD) cycles (Krishnan et al., 1999). The olfactory EAG rhythm is driven by a peripheral circadian clock in antennal olfactory sensory neurons, persisting under constant darkness (DD) (Krishnan et al., 1999; Tanoue et al., 2004). The rhythm is based on the molecular oscillatory mechanism and is abolished in *per* and *tim* null mutants (Krishnan et al., 1999). The circadian rhythms in olfactory EAG responses and clock gene expressions were both eliminated in cry^b mutant flies (Stanewsky et al., 1998; Krishnan et al., 2001). One might argue that the loss of rhythm is derived from desynchronization among constituent clock cells caused by a loss of photic entrainability. However, it is more likely that CRY serves as an essential component for the oscillatory machinery (Krishnan et al., 2001; Levine et al., 2002; Collins et al., 2006). CRY may function as a transcriptional repressor together with PER, because overexpression of *cry* and *per* repressed CLK-CYC activity in the compound eyes. The function of CRY as a transcriptional repressor was also confirmed in cultured cells (Collins et al., 2006). Thus, CRY's repressor activity explains why most peripheral clocks lose their oscillation in cry^b mutants (Levine et al., 2002).

Cry Serves only as a Photoreceptor

In some peripheral rhythms, CRY seems to serve only as a photoreceptor for photic entrainment of the clock. One good example is cuticle deposition rhythms. In *Drosophila*, cuticle deposition rhythmically occurs in the endocuticle of the furca, an apodemata in the thorax, and the rhythm is controlled by a peripheral circadian clock residing in epidermal cells (Ito et al., 2008). The cuticle deposition rhythm in furca was entrained to LD cycles even when the thorax was cultured *in vitro*, suggesting that the photic entrainment system is independent of the brain and resides in the thorax. In cry^b mutants, the rhythm was not entrained to LD cycles. The entrainability was rescued by the overexpression of *cry* throughout whole body of cry^b mutants. Interestingly, not only cry^b, but also cry^{OUT} knockout mutants completely lacking CRY, exhibit the free-running rhythm of cuticle deposition (Ito et al., 2008). These results suggest that CRY only functions as a photoreceptor and not as a component of the clock machinery in the cuticle deposition rhythm.

A similar function of CRY is seen in the prothoracic gland (PG). Fruit fly adults emerge from the pupal case around dawn (Konopka and Benzer, 1971), when high humidity is thought to protect newly emerged flies from desiccation (Pittendrigh, 1954). This eclosion behavior is a once-in-a-lifetime event, but it occurs rhythmically in a population of flies at different developmental stages. The timing of eclosion is set by the circadian system, which consists of two oscillators: one in the LNv and another in the PG (Myers et al., 2003). Myers et al. (2003) suggested that CRY plays an important role as a core component of oscillatory machinery in the PG because the eclosion rhythm was abolished in cry^b mutants. However, subsequent studies yielded completely opposite results (Mealey-Ferrara et al., 2003; Dolezelova et al., 2007): the rhythmic eclosion was observed even in cry^b and cry^0 (knockout of *cry*) flies both in LD cycles and DD. Therefore, as for the cuticle deposition rhythm in furca, CRY seems not to be involved in the oscillatory mechanism of the clock in the PG. This has been confirmed by molecular studies (Emery et al., 1997; Morioka et al., 2012). The *per* transcript rhythm persisted in the

TABLE 1 | Peripheral clocks in *Drosophila melanogaster.*

Location of clock		Output rhythm/relevant physiology	Relationship to the central oscillator*	References
Chemosensory hairs	Antenna	–	A	Plautz et al., 1997
	Proboscis	–	A	Plautz et al., 1997
	Legs	–	A	Plautz et al., 1997
	Wing margin	–	A	Plautz et al., 1997
Excretory organs	Malpighian tubules	–	A	Giebultowicz and Hege, 1997; Giebultowicz et al., 2001
Digestive organs	Alimentary canal (esophagus, crop, proventriculus, hind gut, and rectum)	–	–*	Giebultowicz et al., 2001
Reproductive organs	Sparmathecae (female)	–	–*	Giebultowicz et al., 2001
	Paraovaria (female)	–	–*	Giebultowicz et al., 2001
	Testis base (male)	Sperm release	–*	Giebultowicz et al., 2001; Beaver et al., 2002
	Seminal vesicle (male)	Sperm release	–*	Giebultowicz et al., 2001; Beaver et al., 2002
	Ejaculatory ducts (male)	Sperm release	–*	Giebultowicz et al., 2001; Beaver et al., 2002
Visual system	Retina (compound eyes)	Electroretinogram	–*	Chen et al., 1992
Sensory neurons	Antenna (antennal neurons)	Olfaction rhythm	A	Krishnan et al., 1999; Tanoue et al., 2004
	Proboscis (gustatory receptor neurons)	Rhythms in gustatory physiology and behavior	A	Chatterjee et al., 2010
Epidermis	Epidermal cells	Cuticle deposition rhythm	A	Ito et al., 2008
Secretory cell	Oenocytes	Sex pheromone synthesis and emission	B	Krupp et al., 2008, 2013
Energy metabolic system	Fat body	Feeding rhythm	Possibly A	Xu et al., 2008, 2011
Endocrine system	Prothoracic gland	Eclosion rhythm	C	Emery et al., 1997; Myers et al., 2003; Morioka et al., 2012

** See **Figure 1B**.*

(-) Oscillations for clock protein or clock gene expression are observed. Final output rhythm is not yet determined.

(–) unknown.*

PG under DD when the central nervous system (CNS)-ring gland (RG) complex containing the PG was cultured. The *per* transcript rhythm and oscillation of TIM were both intact in cultured CNS-RG from cry^b mutant flies. However, the TIM oscillation seemed to free-run in PG cells isolated from a cry^b mutant kept under LD cycles (Morioka et al., 2012). Therefore, in the PG, CRY may play a role in the entrainment of TIM oscillation to LD cycles. Interestingly, *cry* expressed in the PG does not affect *per* oscillation. The *per* transcript levels in the PG increased when the cultured CNS-RG was exposed to 12 h of light. This response of *per* depended on light input from CRY via the CNS, because the response was eliminated by cry^b mutation, isolation of PG from the CNS, and blocking synaptic inputs from the CNS. The expression of *cry* transcripts was quite low in the PG compared with the brain and MT (Morioka et al., 2012). Taken together, it is likely that only a small amount of CRY is expressed in the PG and plays a role in the photic entrainment of TIM oscillation, but is not involved in the oscillatory machinery or the photic entrainment of *per* oscillation in the PG.

HETEROGENEITY IN CIRCADIAN ORGANIZATION IN THE PERIPHERY

Although most peripheral oscillators can maintain their oscillation under *in vitro* culture conditions, the oscillations may not perfectly represent those *in vivo* where they may be influenced by various factors including those from the central clock. This issue has been addressed by several studies to date (Krishnan et al., 1999; Giebultowicz, 2000; Ivanchenko et al., 2001; Ito et al., 2008; Krupp et al., 2008, 2013; Morioka et al., 2012), and various relationships between the central and peripheral oscillators have been reported (**Figure 1B**).

Peripheral Clocks Are Independent of the Central Clock

The Malpighian tubules contain a peripheral clock (Giebultowicz and Hege, 1997) (**Figure 1Ba**). The peripheral oscillator in MT is cell-autonomous and entrained to environmental light without

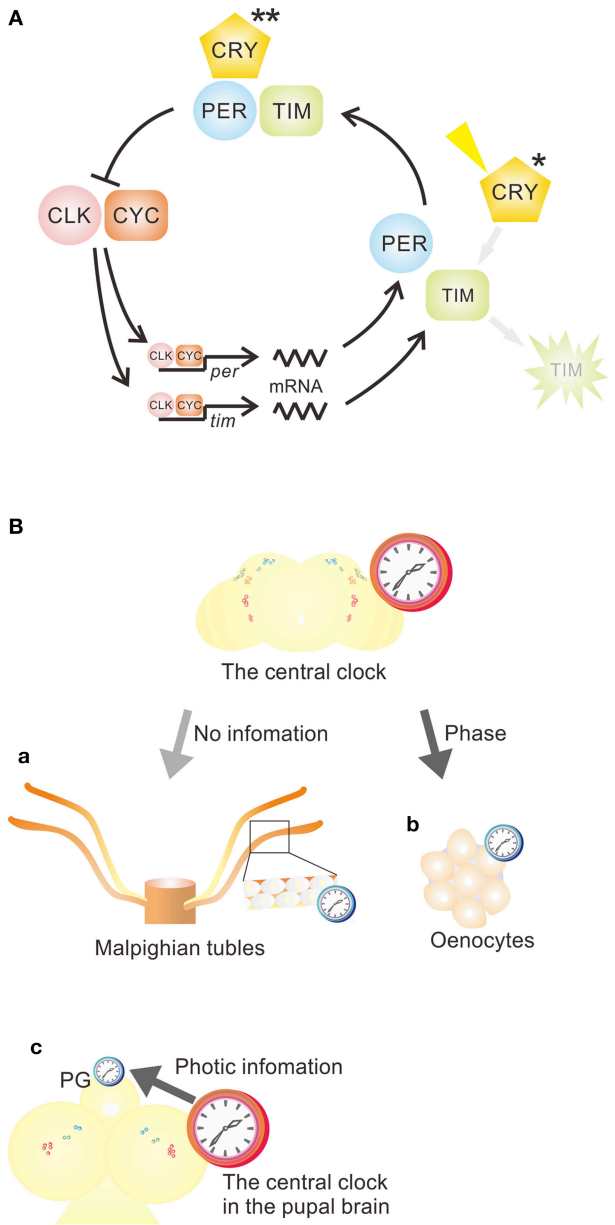

FIGURE 1 | Function of CRY in peripheral circadian clocks and the relationship between central and peripheral clocks in *Drosophila melanogaster*. (A) The functions of CRY vary in peripheral circadian clocks. In most peripheral circadian systems, CRY functions as a photoreceptor (*) and a core component (**) of the clock. However, CRY acts as a photoreceptor (*), but not as a core component of the clock, in the epidermis, which controls cuticle deposition rhythm, and in the prothoracic gland (PG). CLK, CLOCK; CRY, CRYPTOCHROME; CYC, CYCLE; *per*, *period*; *tim*, *timeless*. **(B)** Various relationships between central and peripheral clocks. **(a)** Most peripheral oscillators are independent of the central clock. **(b)** Some peripheral oscillators, such as oenocyte oscillators, are a slave to the central clock, receiving phase information to maintain an appropriate phase relationship to the central clock. **(c)** Some peripheral oscillators, such as those in PG, receive light and temporal signals from the central clock to drive oscillation and coordinate molecular oscillation. See **Table 1** for more examples.

any cue from the central clock (Hege et al., 1997). Independency of the MT oscillator from the central clock was clearly revealed by Giebultowicz et al. (2000). They showed that the original phase of TIM oscillation of MT is maintained in DD when the MT is transplanted into the abdomen of flies previously entrained to antiphase LD cycles.

The antennal circadian clock is also independent from the central clock. The olfactory rhythm driven by the antennal clock was intact even when LNvs, the central clock neurons, were ablated or disrupted (Krishnan et al., 1999; Tanoue et al., 2004). However, the rhythm was lost in flies where the clock oscillation was disrupted only in olfactory sensory neurons (Tanoue et al., 2004). Thus, the peripheral clock in antennae is completely independent from the central clock.

The clock in the fat body of *Drosophila* is another example. It can be entrained independently of the central clock via a restricted feeding schedule (Xu et al., 2011). Interestingly, it has effects on fly metabolism that oppose the effect of the central clock: flies with a genetically disrupted fat body clock show increased food consumption, reduced levels of energy storage, and a higher sensitivity to starvation, whereas opposite responses are observed in energy storage and starvation when the central clock is disrupted (Xu et al., 2008).

Peripheral Oscillator Is a Slave to the Central Clock

Krupp et al. (2008) demonstrated that the circadian clock phase in oenocytes, which regulate pheromone production, is regulated by the central clock. The core clock genes, *per*, *tim*, *Clk*, and *cyc*, showed cyclic expression in LD cycles and DD in oenocytes. These cyclic expressions are abolished in per^0 mutants in DD and in *per*7.2:2 transgenic flies, which only have PER expression in a subset of clock neurons in the brain, but not in peripheral tissues. The results suggest that oenocytes contain a *per*-dependent peripheral clock. The phase of clock gene expression is affected by PDF signaling: it is altered when PDF signaling is disrupted in mutants lacking PDF or a PDF receptor. In those mutants, however, clock gene expression is robustly rhythmic as in wild-type flies and the phase relationship among clock genes is maintained as normal (Krupp et al., 2013). This suggests that the peripheral oscillator in oenocytes is a slave oscillator and its phase is modulated by the central clock, although oscillation itself is maintained independent of the central oscillator (**Figure 1Bb**).

Peripheral Oscillator Is Driven By the Central Clock

The eclosion timing is controlled by a circadian system that consists of two hierarchically organized oscillators located in LNvs and PG, respectively (Myers et al., 2003; Morioka et al., 2012) (**Figure 1Bc**). The targeted disruption of either of these two circadian oscillators using *tim* overexpression renders the eclosion arrhythmic. The eclosion rhythm and molecular oscillation of TIM in PG are also diminished when LNvs are ablated (Myers et al., 2003). These results suggested that both

LNv and PG clocks are necessary for eclosion rhythm and that the PG clock is a slave oscillator driven by the LNv clock. To further dissect this relationship, Morioka et al. (2012) observed the clock gene transcript rhythm and post-transcriptional rhythm in PG *in vitro* and found that the PER oscillation of PG clock receives light information from the central clock, but TIM oscillation does not. Interestingly, TIM maintains its oscillation, but PER does not in PG under DD, although both molecular oscillations are robust under LD cycles (Myers et al., 2003; Morioka et al., 2012). The control from the CNS may contribute to maintaining the robust coordinated oscillations of PER and TIM, which otherwise are dissociated from each other. Thus, the oscillator in *Drosophila* PG is governed by the central clock to a large extent.

CONCLUSION AND FUTURE DIRECTIONS

In early studies, the peripheral oscillators were suggested to be cell autonomous, directly light entrainable, and independent of the central oscillator (Plautz et al., 1997; Giebultowicz, 2000). As described above, new lines of evidence have clearly demonstrated that the oscillatory machinery and degree of independence from the central clock vary among the peripheral clocks. Why and how the peripheral circadian systems have diversified within a species is an open question. Unfortunately, no good answer to this question is currently available. In particular, the function of CRY as a repressor is the most challenging issue in this field. Mammals contain two CRYs, mCRY1 and mCRY2, both of which function as core clock components (Okamura et al., 1999; van der Horst et al., 1999). Many insects also contain two CRYs including *Drosophila* type dCRY and mammalian type mCRY. The former is suggested to be a blue light photoreceptor and the latter to be a transcriptional repressor (Zhu et al., 2005, 2008). *Drosophila* have only one CRY (dCRY) in the genome, which functions as a photoreceptor in the central circadian system (Stanewsky et al., 1998; Helfrich-Förster et al., 2001), but as a transcriptional repressor in the core clock machinery of some peripheral circadian systems (Collins et al., 2006). How the same molecule acts differently in the central and peripheral clocks should be elucidated in future studies. Interestingly, hymenopteran species, including honeybees, only contain mCRY in their genome (Rubin et al., 2006; Zhan et al., 2011). Because hymenopteran species lack *tim* gene in their genome, mCRY is suggested to function as a partner of PER (Rubin et al., 2006). The lack of dCRY might be related to a loss of TIM, because dCRY mediates light-dependent TIM degradation that is required for clock entrainment. It is thus likely that an ancestral insect had both dCRY and mCRY, and that during the course of evolution, some species retained both, while others lost either dCRY or mCRY (Yuan et al., 2007).

The difference between central and peripheral clocks can be also characterized by cell-to-cell communication. The central clock consists of a network of neurons communicating through peptidergic neurotransmitters and maintains its oscillation in various environmental conditions (Yao and Shafer, 2014), whereas communication in the peripheral clock may be variable in organs/tissues and the synchrony among cells is rapidly lost under constant conditions (Plautz et al., 1997; Morioka et al., 2012). Thus, in the latter, synchrony is largely maintained by environmental factors (Plautz et al., 1997; Giebultowicz et al., 2000) or by neuronal or humoral signals from the central clock (Morioka et al., 2012). Detailed molecular studies are necessary to understand the variability and importance of cellular communication in peripheral clock tissue.

Another important issue is the relationship between central and the peripheral clocks, which may be required to temporally optimize local physiology in a tissue-dependent manner. As discussed above, peripheral oscillators can be classified into three types in *Drosophila*: independent of, slave to, and driven by the central clock. Independent peripheral oscillators maintain their own phase, whereas the latter two need to be modulated by the central clock to temporally coordinate physiological and behavioral activity. Therefore, if control from the central clock is through humoral factors, such as ecdysteroids and PDF (Krupp et al., 2013; Uryu et al., 2015), it may be not be uniform, which might be attributable to different oscillatory mechanisms of peripheral clocks. Future studies are needed to examine the possible feedback from the peripheral oscillator to the central clock, which has already been shown in mammalian clock systems (Mohawk et al., 2012).

Although most of the studies on phase control of peripheral circadian clocks have focused on the role of light and the central oscillator, we should pay attention to environmental cues other than light. Obviously the peripheral oscillators utilize temperature changes as time cue (Zeitgeber) in addition to light. Interestingly, the temperature entrainment ability of the peripheral clock is eliminated in flies lacking *no receptor potential A* (*norpA*) encoding phospholipase C (Ito et al., 2011). Although *norpA* mediates thermosensitive splicing of *per* in the central clock (Majercak et al., 2004), its role in peripheral clocks remains to be elucidated. Another important Zeitgeber may be feeding (Xu et al., 2011): the detailed mechanism by which feeding entrains the peripheral oscillator should also be elucidated. Because the precise coordination of amplitude and phase of all clocks is essential for the well-being of animals (Hastings et al., 2003), it remains challenging to explore how multiple circadian clocks in the body are coordinated through entrainment by Zeitgebers and central-peripheral interactions.

AUTHOR CONTRIBUTIONS

CI and KT developed the concept for this mini-review and CI prepared the early draft, including the figure and table. CI researched the literature for the key papers used in this mini-review. KT developed the early draft prepared by CI.

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"The Environment is Everything That Isn't Me": Molecular Mechanisms and Evolutionary Dynamics of Insect Clocks in Variable Surroundings

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Circadian rhythms are oscillations in behavior, metabolism and physiology that have a period close to 24 h. These rhythms are controlled by an internal pacemaker that evolved under strong selective pressures imposed by environmental cyclical changes, mainly of light and temperature. The molecular nature of the circadian pacemaker was extensively studied in a number of organisms under controlled laboratory conditions. But although these studies were fundamental to our understanding of the circadian clock, most of the environmental conditions used resembled rather crudely the relatively constant situation at lower latitudes. At higher latitudes light-dark and temperature cycles vary considerably across different seasons, with summers having long and hot days and winters short and cold ones. Considering these differences and other external cues, such as moonlight, recent studies in more natural and semi-natural situations revealed unexpected features at both molecular and behavioral levels, highlighting the dramatic influence of multiple environmental variables in the molecular clockwork. This emphasizes the importance of studying the circadian clock in the wild, where seasonal environmental changes fine-tune the underlying circadian mechanism, affecting population dynamics and impacting the geographical variation in clock genes. Indeed, latitudinal clines in clock gene frequencies suggest that natural selection and demography shape the circadian clock over wide geographical ranges. In this review we will discuss the recent advances in understanding the molecular underpinnings of the circadian clock, how it resonates with the surrounding variables (both in the laboratory and in semi-natural conditions) and its impact on population dynamics and evolution. In addition, we will elaborate on how next-generation sequencing technologies will complement classical reductionist approaches by identifying causal variants in natural populations that will link genetic variation to circadian phenotypes, illuminating how the circadian clock functions in the real world.

Keywords: circadian, photoperiod, entrainment, latitude, environment, cline, evolution

INTRODUCTION

The environment is the biotic and abiotic surroundings of an organism and one of the most powerful driving forces behind evolution. Among its many facets, the daily environmental cycles of light and temperature generated by Earth's rotation around its own axis are essential ecological factors shaping a variety of adaptations in living beings. In order to harmoniously follow these periodic changes, most organisms, ranging from bacteria to humans, have evolved a genetic multi-oscillatory timekeeping mechanism known as circadian clock (from the Latin *circa* meaning "about" and *dies* meaning "day"; Pittendrigh, 1993). This clock runs with a period of about 24 h and temporally organizes organismal physiology, metabolism and behavior to resonate with Earth geophysical cycles.

Because Earth axis is tilted approximately 23.4° there are, besides circadian fluctuations of environmental conditions, seasonal changes in day length (photoperiod) and temperature throughout the year (Daan, 2010). While nearly constant close to the tropics, these seasonal variations are striking at higher latitudes and constitute a strong selective force for animals and plants. Being poikilothermic, insects face daily environmental changes as a critical challenge for their survival and reproductive success, which ultimately shaped their adaptation to practically all environments on the planet. As with the circadian clock, this strong selective pressure on virtually all insects shaped a photoperiodic mechanism that interprets changes in photoperiod and temperature over the year in order to modulate metabolism and behavior to enhance survival in adverse conditions (Koštál, 2011).

While the circadian clock in insects modulates daily rhythms of activity/rest, eclosion, mating and feeding (Clements, 1999; Saunders, 2002), the most prominent outcome of the photoperiodic mechanism is the diapause, a programmed halt of development associated with changes in metabolism, physiology, and behavior (Schiesari and O'Connor, 2013). But although the interplay between the circadian and photoperiodic clocks has been widely reported in many insect species (reviewed in Bradshaw and Holzapfel, 2010; Saunders, 2010; Koštál, 2011; Saunders, 2013; Dolezel, 2015), it has been difficult to delineate their limits and overlap for mainly two reasons. The first is historical: while the molecular study of the circadian clock has immensely benefited from the genetic tools available for the insect model organism *Drosophila melanogaster* (see below), the lack of a pronounced seasonal response in this species prevented its use for photoperiodic clock studies (Tauber and Kyriacou, 2001). The second is related to the environment: although seminal behavioral observations in nature were pivotal to disclose general patterns of daily and seasonal activity in many insect species (Clements, 1999; Saunders, 2002), the influence of multiple environmental cues that varied in a seasonal and regional manner such as light, temperature, humidity, moonlight, social factors and even food availability, acted as strong confounding factors that made the characterization of circadian and photoperiodic clocks in nature a difficult task.

To circumvent the high complexity of the natural environment, experiments in the late 1950's/early 60's moved toward the lab, where environmental variables could be strictly controlled and tested separately (e.g., Pittendrigh, 1954). In addition, in controlled laboratory conditions it was possible to test the truly endogenous rhythm of a species by removing virtually all environmental conditions, i.e., placing individuals in constant darkness and temperature. This permitted the characterization of the endogenous circadian clock basic parameters (period and the phase), as well as its correlation with cyclic changes of behavior and physiology. This move, allied to powerful genetic screens in *D. melanogaster* that started in the late 60′s has set this species as the most important model in the study of circadian rhythms. Since then, *Drosophila* has contributed immensely to our knowledge on the molecular underpinnings of the circadian clock, and the role of light and temperature as synchronizing factors (also termed *zeitgebers*—"time giver" in German) to harmonize the clock with the changing environment.

Most of the seminal experiments in *Drosophila* used an artificial regime that roughly resembled daily changes of light and temperature at lower latitudes (12 h of light followed by 12 h of darkness in constant temperatures). However, more recent studies simulating conditions at higher latitudes revealed unappreciated clock plasticity at both molecular and cellular levels (Majercak et al., 1999; Rieger et al., 2003; Shafer et al., 2004). When more recently the experimental system was set outside the lab and the impact of all environmental variables were synchronously tested, the fruit fly behavior showed to be dramatically different from what was reported in controlled laboratory studies (Vanin et al., 2012; Das et al., 2015; Green et al., 2015). These late studies have highlighted that although many remarkable advances in understanding the molecular features of circadian clocks have been achieved inside the lab, only in the dynamic natural environment will the circadian and photoperiodic clocks be completely understood.

Crucially, only in the natural environment the adaptive value of circadian rhythms can be tested. Although traits that are environmentally sensitive are not necessarily adaptive, latitudinal clines in circadian genotypes and phenotypes in *Drosophila* have suggested adaptive circadian aspects in populations from different environments, suggesting the action of natural selection (Costa et al., 1992; Sawyer et al., 2006; Tauber et al., 2007).

In this review we discuss past and more recent studies on the role of the circadian clock in the natural environment and its impact on insect population dynamics, especially in *Drosophila*. We describe relevant findings in laboratory conditions related to the clock molecular machinery and the importance of external cues in clock synchronization and temporal niche determination. We discuss how setting up experiments outside the lab has changed dramatically our understanding of how seasonal environmental changes fine-tune the underlying circadian mechanism, which affects population dynamics and geographical variation in clock genes. Indeed, here we describe the benefits of studying latitudinal clines and stress the importance of proper comparative studies between locations in different continents. Finally, we speculate how recent genomic techniques, integrated with molecular methods

and evolutionary biology, will allow the identification of genomic regions that harbor evolutionarily significant changes associated with the circadian clock and its adaptation to specific environments.

INSECT ACTIVITY IN THE FIELD AND ADAPTATION TO LABORATORY CONDITIONS

The literature on daily activity rhythms of insects in nature, especially among mosquitoes, is extremely extensive and covers a large number of species in many different locations (reviewed in Clements, 1999). For example, in a single study Lewis and Taylor used suction traps to describe the diurnal and nocturnal patterns of flight activity rhythms of 400 insect taxa at 46 different locations (Lewis and Taylor, 1965). This study reported differences in insects' day preferences and activity phases: while most species were diurnal and displayed unimodal behavior (one peak of activity during the day), some as *Drosophilidae* showed bimodal distribution of activity around dawn and dusk. Importantly, the authors noted that environmental factors shaped insect's activity: while light intensity influenced the duration of flight in day-flyers, temperature affected the overall abundance of trapped insects (Lewis and Taylor, 1965). Indeed, the overt rhythm of activity is controlled by the circadian clock, which is continuously modulated by the direct effects of environmental cycles in nature.

The first experimental paradigm to access exclusively endogenous-generated circadian rhythms was to analyze an overt rhythm in constant darkness (de Mairan, 1729), a fundamental concept used until the present days (Pittendrigh, 1993). Roubaud was the first to apply this experimental design to an insect, and observed that *Anopheles maculipennis* was active only in the first 2 h of the night. These flight activity rhythms persisted in constant darkness and free-run under endogenous control with a period of 20–22 h (Roubaud, 1918). Hence, many studies used a system that recorded acoustic signals generated by individual flight activity to describe inter-specific period lengths, effects of light, temperature, insemination and blood feeding in many mosquito species (Jones, 1964; Jones et al., 1967; Clements, 1999). Later, more sophisticated systems based on infrared beam interruptions were developed for *Drosophila* (e.g., Tomioka et al., 1997; Helfrich-Förster, 1998), and commercial solutions made available (http://www.trikinetics.com). The Trikinetics system has been widely used in *D. melanogaster* (e.g., Glaser and Stanewsky, 2005; Sehadova et al., 2009; Kumar et al., 2014) and adapted for non-drosophilid insects such as mosquitoes and sandflies (Meireles-Filho et al., 2006; Rivas et al., 2008; Gentile et al., 2009; Rund et al., 2012). These experimental procedures were important to reproduce the first reports in the wild and to set the basis for tackling the circadian clock from the molecular perspective in controlled laboratory conditions. As the molecular description of circadian clock in most insects is not as well-described as in *Drosophila*, in the next section we will focus our attention on the fly work, while we refer the readers to recent reviews in other species (Bloch, 2010; Reppert et al., 2010; Tomioka et al., 2012; Meireles-Filho and Kyriacou, 2013; Tomioka and Matsumoto, 2015).

THE MOLECULAR CIRCADIAN CLOCK IN *DROSOPHILA MELANOGASTER*

The seminal work of Konopka and Benzer was crucial to identify the genetic basis of circadian clock. They screened chemically mutagenized *D. melanogaster* flies searching for individuals with different circadian phenotypes in constant darkness, and isolated mutations that turned locomotor activity rhythms shorter, longer or totally disrupted compared to wild type flies. These three mutations mapped on same locus, which was named *period* (*per*) and constituted the first behavioral gene identified using mutagenesis (Konopka and Benzer, 1971). Later, after the *per* gene was cloned and characterized, its expression was shown to fluctuate in a circadian manner at the mRNA and protein levels in fly heads (Reddy et al., 1984; Jackson et al., 1986; Hardin et al., 1990).

The circadian clock is based on interlocked transcriptional and translational feedback loops governed by transcription factors (TFs), which not only autonomously co-ordinate gene expression, but are also able to respond to changes in environmental conditions to maintain cellular homeostasis (reviewed in Hardin, 2011). In *Drosophila*, several transcriptional loops are interconnected by the TFs CLOCK (CLK) and CYCLE (CYC; **Figure 1**), which heterodimerize and bind to E-boxes sequences (*CACGTG*) in *cis*-regulatory regions close to the promoter of genes whose expression they control (Hao et al., 1997; Abruzzi et al., 2011; Meireles-Filho et al., 2014). In a first loop, CLK/CYC activates the expression of *per* and *timeless* (*tim*) (Allada et al., 1998; Darlington et al., 1998; Rutila et al., 1998), whose products accumulate in the cytoplasm during early night, heterodimerize and shuttle back into the nucleus to block CLK/CYC activity by forming an inactive multimeric complex PER/TIM/CLK/CYC (Lee et al., 1998, 1999; Bae et al., 2000; Yu et al., 2006). Without CLK/CYC mediated activation *per* and *tim* levels start to decrease, which leads to a decrease in PER and TIM levels that consequently releases CLK/CYC inhibition, allowing a new round of transcription to occur. In the second loop, CLK/CYC activates *vrille* (*vri*) and *Pdp1*ε expression (**Figure 1**; Cyran et al., 2003; Glossop et al., 2003). Although controlled by the same activator, VRI and PDP1 accumulate at different phases and compete with each other to bind on promoter region of *Clk*. Because VRI is a repressor and accumulates earlier than PDP1 (the activator), this lag of abundance promotes *Clk* circadian expression (Cyran et al., 2003; Glossop et al., 2003). In the third loop, CLK/CYC activates *clockwork orange gene* (*cwo*) expression (**Figure 1**), which codes a transcriptional repressor belonging to the basic helix-loop-helix (bHLH)/orange family of TFs. CWO also binds to E-box sequences and therefore competes with CLK/CYC binding to these regions, including its own promoter, as well as *vri* and *Pdp1* ones (Matsumoto et al., 2007). CWO negative regulation directly affects E-box-mediated transcription, contributing to general stability of the pacemaker (Kadener et al., 2007; Lim et al., 2007; Richier

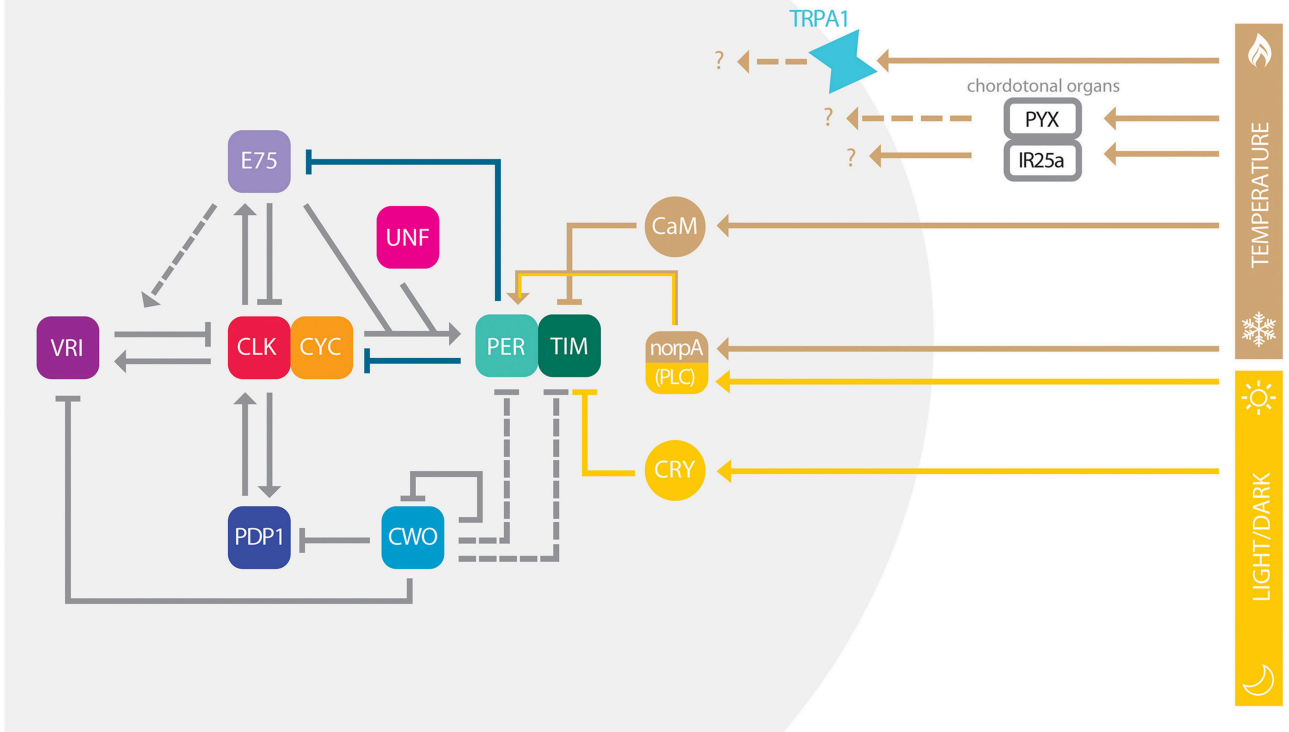

FIGURE 1 | Conceptual model of circadian clock in *Drosophila*. The model depicts multiple interlocked loops, which are centrally governed by the heterodimer CLK-CYC. For simplicity, we do not differ genes from regulatory sequences and proteins, in each case (e.g., PER) these are represented by their names and colored boxes. Arrows indicate positive/enhanced regulation, while blunted ends indicate negative/inhibitory regulation. Gray solid lines depict transcription regulation, while deep blue lines depict protein-protein interactions. Yellow lines show direction of light entrainment pathway, while brown lines depict temperature entrainment pathway. Dashed lines represent pathways that are plausible, but not fully described. While IR25a within the ChO is required for pacemaker synchronization, the roles of TRPA1 and PYX channels in temperature sensing remain to be elucidated. Temperature and light entrainment is also modulated by PLC encoded by *norpA*.

et al., 2008). Recently, two nuclear receptors were implicated in a new feedback loop: *unfulfilled* (*unf*) and *E75* (**Figure 1**). Although the precise molecular mechanism is still unclear, *unf* and *E75* knockdown flies are arrhythmic. Moreover they are both expressed in the circadian pacemaker neurons and collaborate to enhance CLK/CYC-mediated transcription of *per*, but curiously not *tim* (Beuchle et al., 2012; Jaumouillé et al., 2015). E75 alone binds and represses *Clk* expression and enhances VRI repression, suggesting that this nuclear receptor is important to modulate *Clk* regulation alone or together with VRI (**Figure 1**). In adittion, PER suppresses E75 activity, revealing a new role for PER as a de-repressor for *Clk* transcription (Kumar et al., 2014).

An emerging picture from the *Drosophila* circadian network is that as more factors are found to participate in the regulatory circuit, gene functions start to overlap (**Figure 1**), creating the robustness necessary for system stability across different environmental variables. Nevertheless, the stable circadian pacemaker is able to integrate multiple intra- and extra-cellular cues through TFs to produce specific patterns of gene expression necessary for physiological and behavioral responses. For example, the recently identified UNF and E75 belong to the class of nuclear receptors that have their activity modulated by small ligands. Although it is still not know which molecule binds UNF, E75 is known to bind heme and respond to gas (Reinking et al., 2005). E75 therefore might serve to couple the circadian clock with variable environmental CO levels or other ligands. Future mechanistic studies on clock TFs might elucidate how they sense signals from the environment to coordinate cellular responses of the circadian clock.

LIGHT AND TEMPERATURE ENTRAINMENT

The endogenous clock is self-sustained but able to use external cues to adjust to a proper time in synchrony with the environment. The major agents of entrainment are light/dark cycles and temperature fluctuations. Insects use several structures and molecules to receive light and temperature inputs and pass it through to clock pacemaker.

In *D. melanogaster* a genome-wide expression analysis showed hundreds of genes responsive to light and temperature, some of them affected through photo and thermoreceptors within the circadian clock, while others were directly influenced by light or temperature independently (Boothroyd et al., 2007). Interestingly, temperature can affect a higher number of transcripts compared to light, which demonstrates how this environmental agent is important to entrainment. Indeed, low temperatures enhance the splicing of an intron in *per*, which advances phases of *per* mRNA and protein levels (Majercak et al., 1999). Consequently, flies in cold days are more diurnal

than normal, which is an important seasonal adaptation during the winter adjusting the behavior to warmer temperatures. Similarly, an isoform of *tim*, termed tim^{cold} is dominant under low temperatures and in turn contributes to increase the overall *tim* transcript levels (Boothroyd et al., 2007). In fact, *tim* and *per* are both important for seasonal adaptation related to light and temperature due to polymorphic differences displayed among populations (see more details below).

The molecular mechanism of light/dark cycles entrainment is well-characterized in many species. In *Drosophila* this is mainly achieved by the flavoprotein *Cryptochrome* (CRY); cry^{b} mutant respond barely to light pulses and is still rhythmic under constant light, a condition that leads wild type flies to arrhythmicity (Stanewsky et al., 1998; Emery et al., 2000; Kistenpfennig et al., 2012). Light induces a conformational change that activates CRY, which then interacts with TIM and conduct it to proteasome degradation through the E3 ligase protein JETLAG (Suri et al., 1998; Yang et al., 1998; Ceriani et al., 1999; Naidoo et al., 1999; Lin et al., 2001; Busza et al., 2004; Koh et al., 2006; Peschel et al., 2006). In addition BRWD3, a substrate receptor for CRL4 (cullin 4 ring finger E3 ligase), has also been implicated in light induced CRY ubiquitination and degradation (Ozturk et al., 2013). As TIM degradation occurs only during daytime, TIM levels are restricted to the night. As PER is unstable without TIM, PER levels decrease in early morning and CLK/CYC inhibition is released, which is crucial to activate important output genes and dictate diurnal fly activity. In addition to CRY, light entrainment in flies is a result of multiple photoreceptors acting in synergy, since the entrainment by light is completely impaired when all external and internal photoreceptors are eliminated (Helfrich-Förster et al., 2001).

Not surprisingly, temperature entrainment depends on a functional clock since per^{0} mutants merely respond to temperature cycles (Wheeler et al., 1993; Yoshii et al., 2005). While in flies most organs independently entrain their local clocks with temperature cycles, the brain mostly relies on inputs from external thermal sensory structures such as the chordotonal organ (ChO; Sehadova et al., 2009; Simoni et al., 2014). It has been recently shown that the *pyrexia* (*pyx*) and the *Ionotropic Receptor 25a* (*IR25a*) genes are expressed in the ChO but exert different functions, the first being important to synchronization at lower temperatures and the other to perception of small temperature cycles (Wolfgang et al., 2013; Chen et al., 2015). In addition the *TrpA1* gene, which is expressed in clock and non-clock neurons in the *Drosophila* brain, might be important in the temperature-dependent regulation of afternoon siestas (**Figure 1**; Lee, 2013; Lee and Montell, 2013; Das et al., 2015; Green et al., 2015). At the molecular level temperature increases intracellular Ca^{2+} levels, which in turn triggers CALMODULIN(CaM)-mediated degradation of TIM by the Small Optic Lobe (SOL) protease (Tataroglu et al., 2015). As for the CRY-dependent light TIM depletion, temperature-mediated TIM degradation also resets the clock, showing that both light and thermal input pathways make use of TIM to synchronize the clock. Similarly *norpA*, which encodes phospholipase C, was also shown to be crucial not only for *Drosophila* phototransduction but also for temperature entrainment (Collins et al., 2004; Majercak et al., 2004; Glaser and Stanewsky, 2005), suggesting that light and temperature act not only independently but also in an integrated manner to compensate and reinforce the adaptation to a specific temporal niche. However, inside the laboratory, even when in combination, multiple *zeitgebers* are artificial and cannot reflect the complexity of the natural environment.

ENTRAINMENT AND NON-CIRCADIAN INFLUENCE OF ENVIRONMENT IN INSECT'S RHYTHMICITY

A pure expression of the endogenous circadian clock is generated in constant conditions. However, an animal's daily behavior is an interplay between the circadian pacemaker and immediate responses to environmental changes, which can be very different from what is observed inside the lab.

In some species there is a reasonable concordance in locomotor activity rhythms between the lab and nature. For example, the sandfly *Lutzomyia longipalpis* has a major peak of activity soon after lights off under 12:12 LD cycles (Meireles-Filho et al., 2006; Rivas et al., 2008) that persists in several other photoperiodic regimes (Rivas et al., 2014). This is in good agreement with the hourly abundance of eight wild species of sandflies captured in forested areas, which peak in abundance just after the sunset during summer (Guernaoui et al., 2006). Nevertheless, during autumn sandflies are scarcer at night and mostly peaked a little later (2 h after sunset), possibly influenced by differences in temperature and humidity levels between seasons (Guernaoui et al., 2006). Similarly, there are differences in overall activity levels depending on the lunar cycle: *Lutzomyia intermedia* and *Lutzomyia whitmani* females are more active during first, last quarters and full moon nights compared to moonless nights (Souza et al., 2005), a phenomenon also observed in crepuscular mosquitos (Provost, 1958; Bidlingmayer, 1964; Charlwood and Jones, 1980). Overall, these results suggest that even small variations in environmental conditions are able to influence overt activity rhythms.

Strikingly, there are cases where the "natural" behavior of a given species can be very different from what it is observed in the laboratory. For example, hamsters are crepuscular in the wild but nocturnal in laboratory (Gattermann et al., 2008), and mice that are strictly nocturnal in the laboratory, may be partially or completely diurnal in the field (Daan et al., 2011). As individuals in nature are daily influenced by a complex environmental scenario that varies seasonally and geographically, the temporal niche (diurnal, crepuscular, nocturnal) observed is a mixture of immediate responses to environmental changes and clock-controlled processes. Indeed, nocturnal animals switch the temporal niche to diurnal in conditions of low temperature or food restriction, which avoid energy expenditure in cold nights (Hut et al., 2013; van der Vinne et al., 2014, 2015). Being poikilothermic, insects exposed to different temperature levels can also change the onset activity and diurnal activity proportion (Lazzari, 1992; Majercak et al., 1999; Rivas et al., 2014) using the same adaptive strategy of homoeothermic organisms. In this sense, variable environmental cues can not only act on clock synchronization, but also produce a direct effect on behavior independently of it, a phenomenon termed "masking" (reviewed by Mrosovsky, 1999). Masking can inhibit or stimulate activity

and disguise the clock-controlled phase of behavior. For example, *Drosophila malerkotliana* is diurnal under low but nocturnal under high light intensities (Sharma et al., 2012), a specific kind of masking called "paradoxical" (Mrosovsky, 1999), which is reinforced by the combined effects of bright light and high temperature (Sharma et al., 2012). Therefore, both masking and entrainment act in combination with the central clock to fine-tune overt daily rhythms of activity in nature.

In an attempt to elucidate how the molecular circadian clock operates in nature, numerous recent studies simulated different environmental conditions in the laboratory. One environmental component that is highly variable and known to affect the activity of insects, both through entrainment and masking, is light intensity. Circadian clocks are highly light-sensitive and can be influenced even by low irradiances that occur at dawn, dusk and moonlight (Rieger et al., 2007). In controlled laboratory conditions, *D. melanogaster* shows two peaks of locomotor activity around dawn and dusk (morning "M" and evening "E" peaks, respectively) and a period of less activity in the middle of day, a behavior that was termed the "siesta" (Hamblen-Coyle et al., 1992; Wheeler et al., 1993; Helfrich-Förster, 2000). In the laboratory fruit flies have a preference for low light intensities (between 5 and 10 lux) that allow the expression of different behavioral phenotypes, such as resting, grooming and feeding (Rieger et al., 2007). In this respect, exposure to artificial light similar to quarter-moonlight (0.03 lux) changes the phase of the endogenous clock, consequently shifting both peaks of locomotor activity into the night (Bachleitner et al., 2007). Importantly, clock mutants were also able to switch their temporal niche, suggesting that light bypassed the circadian clock to directly modulate fly activity patterns (Kempinger et al., 2009). Indeed, the compound eyes were recently shown to drive this masking effect in a clock-independent manner, both in moonlight perception (Kempinger et al., 2009; Schlichting et al., 2014) and in the timing of M and E peaks under natural-like conditions (Schlichting et al., 2015a). Therefore, under semi-natural conditions of moonlight or gradual light transitions at dawn and dusk (which mimics twilight), flies use mainly the visual system to determine locomotor activity peak times, where twilight has a more dominant role in fruit fly behavior than moonlight (Rieger et al., 2007; Schlichting et al., 2015b).

These somehow counter-intuitive results highlighted the difficulties associated in studying the combined effects of the environment in locomotor activity measurements inside the lab and encouraged more "natural" set ups.

NEW PERSPECTIVES AND STUDIES IN SEMI-NATURAL CONDITIONS

The simplicity of the locomotor automated system used by most fly labs allowed its adaptation to outdoor areas, once shaded places and rain protection were provided. Recently, Vanin et al. (2012) applied this set up in two different natural locations, Leicester, UK and Treviso, Italy and measured *D. melanogaster* locomotor activity through different seasons. Surprisingly, in contrast to what was expected from laboratory studies, flies were notably diurnal in the wild, with crepuscular activity not reaching 25% of the total amount recorded. In addition, disagreeing with the "siesta" observed in laboratory measurements at high temperature, the authors observed a major activity peak at midday during summer, which they termed the "A" (afternoon) peak. Importantly, unlike "A" and "E" peaks that are clock-modulated, only the tim^0 mutant showed temperature independence in morning onset activity, suggesting that the "M" component is driven by twilight-dependent temperature cycles and not the circadian clock. Finally, temperature was more important for entrainment than light/dark cycles since locomotor behavior from flies in Leicester (UK) and Treviso (Italy) were quite similar, despite a considerable difference of daytime length between these regions in summer (Vanin et al., 2012).

As noted previously, given that environmental conditions are highly dependent on geographical location, comparisons between experiments performed in different regions should be made with caution, as it is virtually impossible to know all variables playing a role in nature. Nevertheless, following this study other groups used the same experimental procedure to measure fly locomotor activity outside the laboratory and reached similar observations. In Würzburg, Germany, Menegazzi and colleagues found only minor differences regarding the onset of the "E" peak in per^{01} mutant flies, which was delayed compared to wild-type flies in this study (Menegazzi et al., 2012) but anticipated in Vanin et al. (2012). Similarly, most of the previous findings were also observed in a region of South India, except that the authors of this study mistakenly suggested that the "A" peak was an artifact caused by the experimental procedure from Vanin et al. (2012) (De et al., 2013). Indeed, recent results confirmed the existence of the "A" peak with different methodological approaches (glass tubes used in TriKinetics monitors and in open-field arenas), in temperate and sub-tropical regions and even in De et al. (2013)'s own data, which overall suggests that the "A" peak is a clock-modulated escape response (Green et al., 2015). Although it is striking that these reports reached very similar conclusions despite of differences in latitude where these studies took place (52°N for Leicester, 45°N for Treviso, 49°N for Würzburg, and 12°N for South India), future work should aim to use even more natural conditions as in nature flies experience social, auditory, olfactory, and gustatory cues that influence their activity pattern. Altogether, these results show that even under different environmental conditions the circadian clock is able to operate in robust harmonic configurations.

Early findings in laboratory conditions had shown that even small differences of 2–3°C were able to phase-shift the *Drosophila* rhythmic locomotor behavior (Wheeler et al., 1993). In the wild, temperature cycles were especially important on the determination of the "A" component, which was shown to be dependent on the *TrpA1* channel expressed in *TrpA1*-expressing neurons other than the canonical clock ones (Das et al., 2015; Green et al., 2015). At the pacemaker level, *norpA* splices the 3' intron of the *per* mRNA transcript in a temperature-dependent manner, changing PER levels that consequently will advance or delay the circadian phase (Majercak et al., 1999, 2004; Collins et al., 2004). In semi-natural conditions, *per* mRNA cycling in fly heads is observed only in summer, while *tim* cycles robustly throughout the year (Montelli et al., 2015). Accordingly, PER but not TIM abundance follow

seasonal changes in the fly brain (Menegazzi et al., 2013), confirming the importance of PER in interconnecting seasonal environmental changes to behavioral responses. Overall, these results highlight an interesting anatomical difference between light and temperature entrainment in flies: while light can activate CRY in a cell autonomous manner, temperature reaches the brain indirectly probably through several different channels expressed in peripheral organs.

Given the pivotal importance of temperature to dictate *Drosophila* daily activity in nature, these results should encourage further experiments upon the role and hierarchy of each thermoreceptor in regimes with conflicting gradual light and temperature cycles, especially among wild populations that show latitudinal clines.

GENETIC AND PHENOTYPIC VARIATION ACCORDING TO GRADUAL ENVIRONMENTAL CHANGES

Evolutionary studies indicate species as a group of organisms preserving their uniqueness that are often adapted to their local environment. The interaction between species and environment raises interesting questions. How can different sympatric organisms, such as plants and animals be so divergent if they are ruled by the commands of the same environmental conditions? In species showing broad range distribution pattern (covering large geographical transects such as latitude, altitude or longitude), how can allopatric individuals conserve their taxonomic identity if they are governed by the commands of different environmental conditions?

For many years, biologists have been trying to understand evolutionary forces influencing genetic variation within and among species (Lewontin, 1974; Hughes, 2007; Mitchell-Olds et al., 2007). It has been a long search to connect this genetic variation with variation in phenotypes and fitness within natural populations. One good possibility to tackle this problem is to study sample variation along geographic transects, which provides great benefit when compared with studies performed on patchy samples. Clines can be defined as predictable geographic gradients in a genotype or a phenotype that can be measurable (Endler, 1977) and can be replicable to a degree that variation sampled from patchy landscapes can not. The main benefits of studying clines include the possibility of parallel adaptation and the attenuation of some of the confounding effects of demography. A good example is that a cline along a coastal latitudinal transect can be potentially replicated on multiple continents, providing evidence of parallel adaptation. Indeed, one further classic way of differentiating between natural selection and drift in the generation of a latitudinal cline is to study the polymorphism in different continents (Oakeshott et al., 1981).

In *Drosophila* and other species, several studies were performed considering phenotypic, genetic, and genomic gradual change variation over the geographical range of the species distribution (reviewed by Kyriacou et al., 2008; Yerushalmi and Green, 2009; Costa and Stanewsky, 2013; Hut et al., 2013; Adrion et al., 2015). For example, *Drosophila littoralis* is a latitudinal widespread European species of the *Drosophila virilis* group that has ample genetic variation in photoperiodism (diapause in adults) and circadian rhythmicity (pupal eclosion), with adaptive latitudinal clines in both of them (Lankinen, 1986). Interestingly, it was shown that the adaptively variable gene *loci* are different for photoperiodism and the circadian clock, and that threonine-glycine repeat section of the *per locus* (a strong candidate for clock variability, see Clines in Clock Genes section) was not included in the polymorphism of *D. littoralis* clock genes (Lankinen and Forsman, 2006). Phenotypic differentiation along latitudinal transects has been shown for various traits in *D. melanogaster* and many patterns are recapitulated among continents (Adrion et al., 2015). Nevertheless, *D. melanogaster* shows a number of latitudinal clines in morphological characteristics such as body size (James et al., 1997) or in frequencies of various metabolic genes (Oakeshott et al., 1981, 1983a,b, 1984; Bubliy et al., 1999), suggesting that selection, in the face of considerable drift and migration, has already made its mark. Single molecular markers have also been used to elucidate clinal differentiation and spatial variation in allele frequencies, revealing variations that tracked the clines (reviewed in Kyriacou et al., 2008; Costa and Stanewsky, 2013; Hut et al., 2013). Those studies increased the scientific understanding of local adaptation and the characterization of selective forces determining the high diversity in phenotypes observed in nature.

For a better understanding of latitudinal clines it is important to know the correlation between photoperiod and temperature (Hut et al., 2013). Contrary to temperature oscillations, the photoperiod parameter remains stable over the years, with the duration of the civil twilight being dependent on the latitude (**Figure 2**). Temperature is a parameter that is more affected by the distribution of landmass and sea currents over the globe, causing quantitative space and temporal differences. For instance, due to the ocean Gulf stream that affects climate conditions in Europe, Boston (USA, 42°N) is colder than London (51°N) in winter even though it is lower in latitude. Overall, while temperatures vary considerably over the years, the photoperiod remains stable. Organisms therefore use photoperiod as a proximate factor to tune the annual timing of physiology and behavior to changes in ultimate factors such as temperature

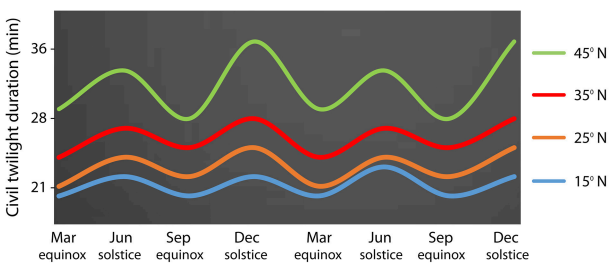

FIGURE 2 | Latitudinal variation in the duration of the civil twilight along two consecutive years. In the abscissa it is shown astronomical events (equinoxes and solstices). Latitude 15°N in blue, latitude 25°N in orange, latitude 35°N in red, latitude 45°N in green.

(Hut et al., 2013). Studies at the molecular level performed for documentation of segregating molecular polymorphisms would benefit more if disentangling complex interacting traits in annual timing. Considering more predictable geographical parameters such as the photoperiod may simplify conclusions of molecular studies performed in latitudinal transects.

PARALLEL CLINE STUDIES PERFORMED BETWEEN CONTINENTS

Evidence for parallel adaptation and identification of commonalities in the genes responding to cline selection have been performed among continents. For instance, North American and Australian eastern coastal clines have been heavily studied for both phenotypic and genetic variation (reviewed in Adrion et al., 2015). The North American cline has been sampled from southern Florida, USA (25°N) to Vermont and Maine USA (44°N). The Australian cline has been heavily sampled from northern Queensland (15°S) to Tasmania (43°S). There is high environmental variation along these clines. High latitude populations on both continents experience lower mean temperatures, greater variance in temperatures across season and reduced UV light exposure (Hoffmann and Weeks, 2007). The latitudinal range in Australia takes into account a large portion of tropical climate whereas the latitudinal cline range in the USA does not. This has to be considered with caution. For a proper parallel adaptation comparison the geographical ranges studied should take into account exactly the same latitudes. Because tropical populations below 25°N in the Americas have been under explored it is unknown if inclusion of theses populations would strengthen or lessen support for parallel adaptation among Australian and North American clines. Another problem is that the highest latitudes observed in Australia cover an island area (Tasmania) and this can bring specific demographic features such as restricted gene flow and inbreeding, which make populations living in such environments particularly prone to the effects of drift. The Southern hemisphere has less land and a wider ocean area compared to the Northern hemisphere. Also the land distribution in the South hemisphere hardly reaches high latitudes. South America is the only continent in the Southern hemisphere where a continuous land latitudinal cline transect can be determined, from low up to very high latitudes (**Figure 3**). Unfortunately almost nothing is known about the clinal genetics and history of South American populations. Although cline studies bring lots of advantages, one has to bear in mind that not all differentiation occurs in parallel among continents. There are cases where patterns are not repeatable among clines. For example, in contrast to the well-defined cline in North America, the incidence of diapause (Lee et al., 2011) and the number of ovarioles (Azevedo et al., 1996) in *Drosophila* display non-linear associations with latitude in eastern Australia.

CLINES IN CLOCK GENES

Clinal variation in circadian genes has previously been identified in a variety of organisms such as salmonids (O'malley and Banks, 2008; O'malley et al., 2010), passerine birds (Johnsen et al., 2007) and plants (Chen et al., 2012; Keller et al., 2012), in addition to *D. melanogaster* (Costa et al., 1992; Sawyer et al., 2006; Tauber et al., 2007; Kyriacou et al., 2008). Moreover, properties of circadian rhythms in the same species living at different latitudes have been useful in examining the correlation of circadian rhythms with the environment, reaching the conclusion that having a circadian system that matches the oscillating environments is adaptive (Yerushalmi and Green, 2009).

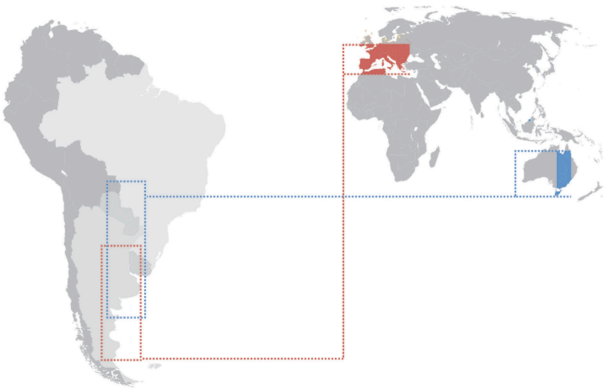

FIGURE 3 | Latitudinal clines in (Thr-Gly)$_{20}$ frequencies studied in Europe (Costa et al., 1992 red) and Australia (Sawyer et al., 2006 blue) and how they would correlate if the considered latitudinal areas were transposed to South America.

One of the best known cline studies in circadian clock genes was performed with the *per* gene. Within PER there is a repetitive region composed of alternating dipeptides of threonine and glycine (Thr-Gly) residues (Yu et al., 1987). In *D. melanogaster*, the repeat is highly polymorphic, both in sequence and length (Costa et al., 1991). Repeat length variation in natural populations within Europe follows a latitudinal cline, so that high frequencies of the (Thr-Gly)$_{20}$ and (Thr-Gly)$_{17}$ alleles are found in Northern and Southern regions, respectively (Costa et al., 1992). Linkage disequilibrium pattern analysis in this region suggested that balancing selection may be operating and this would seem to fit in nicely with the clinal distribution (Rosato et al., 1997). Finally, taken together these two length alleles make up approximately 90% of the natural variation found in Europe. Variants carrying 14 (1%) and 23 (8%) Thr-Gly pairs and very rare variants with 18, 21, and 24 pairs (together counting for 1%) make up the rest. The temperature compensation of the clock differs among the Thr-Gly variants (Sawyer et al., 1997). The (Thr-Gly)$_{17}$ variant has a 24 h cycle at higher temperature, but the period becomes shorter as the temperature is reduced. On the other hand, the (Thr-Gly)$_{20}$ variant shows a period that is not sensitive to temperature change and is on average slightly shorter than 24 h, appearing to be better buffered against temperature swings (Sawyer et al., 1997).

A cline in Thr-Gly polymorphism in the *per* gene in Australian populations of *D. melanogaster* was reported to be weaker than

the European one, and this observation was consistent with the view that natural selection was maintaining the polymorphism in both continents (Sawyer et al., 2006). This cline would be particularly interesting in view of the fact that *D. melanogaster* was introduced to Australia 100 years ago (Umina et al., 2005). However, the existence of clinal variation in the 17 or 20 repeats alleles in Australian *D. melanogaster* populations is a matter of debate (Weeks et al., 2006, 2007; Kyriacou et al., 2007). Nonetheless, it is important to point out that the $(Thr\text{-}Gly)_{20}$ clinal distribution compared between Europe and Australia is in quite different latitude ranges (from 33.40 up to 52.10° of latitude in Europe and from 16.88 up to 42.88° of latitude in Australia). Maybe one of the reasons for the less steep $(Thr\text{-}Gly)_{20}$ cline in Australia was due to the fact the data were not analyzed for the same latitude range when comparing the Thr-Gly distributions in Europe and Australia. **Figure 3** shows the distribution cline ranges studied in Europe (Costa et al., 1992) and Australia (Sawyer et al., 2006), and how the distribution range would not overlap entirely if the collections were performed in South America, for instance. By plotting a distribution graph comparing the Thr-Gly frequencies between Europe and Australia and discarding the more tropical Australian data (latitude below 25°S), it can be observed that the frequencies of $(Thr\text{-}Gly)_{20}$ alleles are as steep in Australia as the frequencies are in Europe (**Figure 4**). This fact confirms that in Australia the trend is toward a greater number of Thr-Gly alleles and higher heterozygosity in the tropical lower latitudes (Sawyer et al., 2006) raising the hypothesis that the relaxed thermal selection of the tropics is more forgiving than the European climate (Kyriacou et al., 2007). Indeed, this hypothesis is supported by studies performed in the Nahal Oren Canyon, Israel, which is a canyon separated by only 200 m where the two neighboring slopes have "tropical-like" or "European-like" microclimates. While the frequencies of $(Thr\text{-}Gly)_{20}$ alleles were higher in the "European" slope, frequencies of $(Thr\text{-}Gly)_{17}$ were higher on the "tropical" slope (Zamorzaeva et al., 2005). Overall these observations suggest that future studies should analyze more temperate latitudes in both continents in order to find parallel adaptations.

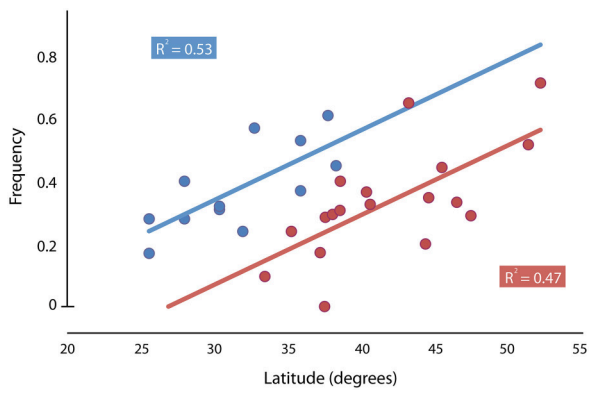

FIGURE 4 | Latitudinal variation of *per* $(Thr\text{-}Gly)_{20}$ repeat length frequency in European (red dots—Costa et al., 1992) and Australian natural populations (blue dots—Sawyer et al., 2006). In order to adjust the analysis for the same latitudes, the tropical Australian samples (latitude <25°) were not considered. Also, samples from the Australian state of Tasmania were not considered as conditions in an island may differ from conditions in the mainland, as well as the selective pressure acting on these flies.

Studies on clines' polymorphisms in circadian genes do not always provide such clear confirmation of the adaptive value of circadian rhythms, as occurs in *Drosophila* with the two common alleles of the *tim* gene (*ls-tim* that expresses both long and short forms of TIM protein and *s-tim* that only gives the short form of the protein). *ls-tim* probably arose in southern Italy <10,000 years ago and causes attenuated photosensitivity of the circadian clock (Sandrelli et al., 2007; Tauber et al., 2007). Unexpectedly, there is a significant latitudinal cline with more *ls-tim* in southern Europe and more *s-tim* in northern Europe. It was suggested that the distribution pattern of *tim* polymorphism reflects the adaptive advantage of the relatively new *ls-tim* allele spreading under directional selection from its place of origin (Tauber et al., 2007). This was confirmed by showing that the latitudinal cline was in fact a distance cline from the point of origin, and that the frequency of the new allele was proportional to the overland distance from southern Italy. The *ls-tim* allele can potentially confer several adaptive advantages in environments at high latitudes, both in winter and summer. First, as in winter the days become colder faster than they become shorter, the relative insensitivity of *ls-tim* to long summer photoperiods allow flies to undergo diapause in long cold days. Second, when flies are exposed to a quasi LL (constant light) in midsummer, a condition that leads them to arrhythmicity (Stanewsky et al., 1998), the relatively reduced circadian response to light allow their clock to keep running under such conditions (Sandrelli et al., 2007; Tauber et al., 2007). Besides *tim*, polymorphisms in the *couch potato* and *insulin-regulated PI3 kinase* genes have also been implicated in diapause expression (Williams et al., 2006; Cogni et al., 2014), and it will be interesting in the future to evaluate if these pathways converge or affect diapause independently. Generally, latitudinal variation seems to be a powerful tool to study circadian mechanisms and photoperiodic responses to reveal selective forces involved in daily and seasonal adaptation (Emerson et al., 2009; Hut and Beersma, 2011).

In the future one can rely on whole genome analyses of populations over a geographical transect to identify both large and fine scale clinal patterns. Regions of the genome that are strongly differentiated between samples can be identified revealing signatures of adaptation. Differentiated regions that overlap among multiple clines can provide evidence of parallel adaptation. It will be promising to check for highly differentiated sites in the genome between two samples in different continents and verify if they might cluster by environment than by continent, providing evidence for the role of the environment in shaping genotypic and also phenotypic traits.

DISCUSSION

Insects make use of the visual system, mechanosensory pathways and thermo and olfactory receptors to perceive the environment.

These structures transduce multiple external cues into an environmentally sensitive gene network, the circadian clock. The circadian pacemaker processes external information within its network and regulates the expression of hundreds of genes, protein stability and protein abundance in order to daily synchronize many aspects of metabolism, physiology, and behavior with the environment.

Environmental oscillations are paramount ecological factors for generation of adaptive traits among several species. In this respect, adaptations to daytime or nighttime activity are dependent not only on direct and effective responsiveness to environmental changes (e.g., bright light intensity or midday high temperatures), but also on anticipating these changes. Thus, the proper resonation of the clock with the environment is of fundamental importance for an organism to adapt to its surroundings.

Clock genes in insects (and other multicelular eukaryotes) encode important factors that modulate hundreds of genes to drive cyclical changes in physiology and behavior. Correlations of circadian rhythms with the environment have been demonstrated in several species, providing mounting evidence that having a circadian system that matches the oscillating surrounds is adaptive (Yerushalmi and Green, 2009). In this sense, clock genes are excellent candidates for acting as speciation genes and can be used to unveil speciation in insects (e.g., Colot et al., 1988; Peixoto et al., 1993; Bauzer et al., 2002; Tauber et al., 2003; Araki et al., 2009; Rona et al., 2010). But as the circadian transcriptome of different insect species depicted that many genes display circadian expression (Ceriani et al., 2002; Ptitsyn et al., 2011; Rund et al., 2011), the exclusive choice of clock genes as candidates for evolutionary studies certainly would tell just part of the whole history. Moreover, some important adaptive traits based on findings in *Drosophila*, such as the molecular basis of temperature compensation, could not be extrapolated for other insect populations, such as mosquitoes and sand flies. For example, the Thr-Gly polymorphism in the *per* gene is pivotal for natural temperature compensation and adaptation of European populations *D. melanogaster* (Costa et al., 1992) but was not found in *per* of *D. littoralis* (Lankinen and Forsman, 2006) and in *per* orthologs from mosquitoes and sand flies (according to our search on vectorbase database—vectorbase.org). This suggests that other mechanisms for temperature compensation in these species might be encoded in other parts of genome and raises an important point that although conserved, the circadian mechanism has species-specific differences that demand dedicated unbiased approaches in order to reveal them.

In this sense, genomic technologies now provide unprecedented access to evolutionary genetics. Natural populations within and across continents that are expected to show considerable level of genetic diversity can be fully sequenced at reasonable costs and genotype-phenotype association studies conducted to access the genomic regions responsible for trait adaptation. Several quantitative genetics studies have been conducted in *D. melanogaster* (Pasyukova et al., 2000; Geiger-Thornsberry and Mackay, 2004; Nuzhdin et al., 2005; Remolina et al., 2012), and also in mosquitos such as *Aedes aegypti* (Bennett et al., 2005; Saavedra-Rodriguez et al., 2008; Reyes-Solis et al., 2014) and *Anopheles gambiae* (Zheng et al., 1997, 2003; Blandin et al., 2009). Comparative studies across different continents regarding latitudinal clines of morphological or circadian related traits will be pivotal to identify genes important for parallel adaptation of populations among continents. In this scenario, South America is quite interesting and in fact there are few reports describing the occurrence of latitudinal clines in some insect species in different regions of this continent (Bubliy et al., 1999; Gilchrist et al., 2004; Rosetti and Remis, 2013).

In this regard, recessive alleles complicate genetic association studies and hence the identification of causal genes in natural insect populations. Alternatively, inbred lines can be used for genetic analysis, particularly in conjunction with genome-wide markers or full genome sequences, to enhance the power for genetic association analyses. For example, in the past, inbred populations have been successfully used in evolutionary genetics studies (Falconer and Mackay, 1996; Lynch and Walsh, 1998). In *D. melanogaster*, the Drosophila Genetic Reference Panel (DGRP) was established as a set of 204 fully sequenced inbred lines (Mackay et al., 2012) that have been used in a number of studies from accessing the genetic basis of sexual gene expression differences (Massouras et al., 2012) to the gut immunocompetence in flies (Bou Sleiman et al., 2015). Therefore, the use of inbred lines in the clock-dependent adaptation studies has the potential to find unique genomic signatures important for *Drosophila* to adapt and predict natural variable surroundings.

It is tempting to recommend that functional genomics should follow the behavioral assays in natural conditions in *Drosophila* and other insect species. In addition to assaying different populations from the same species, multiple comparisons between evolutionary distant species in the same geographical locations might also reveal important phenotypic and genotypic aspects of adaptation related to convergent evolution, and thus provide a more generalist idea of adaptation of temporal niche in species through evolution.

AUTHOR CONTRIBUTIONS

GBSR, LGSRB and ACAMF contributed to the conception, design, draft, and editing of this article.

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