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The apparent seasonal biphenism in *Drosophila suzukii* stems in reality from continuous reaction norms

Hervé Colinet* and Alexiane Kustre



Abstract

The spotted wing drosophila (SWD) is supposed to show only two distinct seasonal phenotypes: the dark, diapausing winter morph (WM) and the light, reproductively active summer morph (SM). It is unclear if these phenotypes result from a true developmental switch or from the expression of extreme phenotypes of continuous thermal reaction norms. This study aims to investigate this question by examining traits across a range of temperatures. Using 12 developmental temperatures (8 to 30 °C), we assessed traits including viability, growth, morphology, cold tolerance, metabolic rate, and ovarian maturation. Gradual increases in temperature induced gradual changes in all these traits, indicating classical nonlinear thermal reaction norms. Low temperatures (14 °C and below) produced flies with extended development, dark color, larger size, increased cold tolerance, reduced metabolism, and delayed oogenesis, characteristic of the WM. Given the months required for emergence and egg maturation at cold, distinct generations of SWD may develop in discrete environments resulting in an apparent biphenism. What appears to be distinct phenotypes (WM and SM) may actually result from continuous thermal reaction norms. This implies the need for precise terminology in SWD. We recommend using terms like 'winter-acclimated' or 'winter phenotype' rather than 'winter morph'.

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Keywords: dormancy; drosophila; phenotype plasticity; polyphenism; thermal reaction norms

1 INTRODUCTION

The Spotted Wing Drosophila (SWD), *Drosophila suzukii*, has invaded both America and Europe and it is still spreading across both continents.¹ This invasive success can be attributed to various factors, including the wide thermal range and plasticity, allowing adaptation to diverse hot and cold climates.^{2,3} Additionally, SWD highly polyphagous nature enables infestations in a variety of cultivated and wild fruits, with a preference for ripe fruits but also exploiting decaying ones.^{4,5} Understanding temporal and spatial dynamics of a pest constitutes the core foundation of any management strategy. For this reason, thermal and seasonal ecology of SWD has been extensively studied over the last decade.^{1,6–8} Yet, uncertainty persists on certain aspects of its overwintering strategy, including whether it exclusively involves adult diapause or quiescence.⁹ Recent works suggest mixed strategies incorporating both adults and developing stages.¹⁰ The lack of clarity about its overwintering strategy has impeded the development of predictive models for assessing its overwintering success.¹¹

Stephen *et al.*¹² published a paper on the winter survival of SWD, in which it was suggested, with reference to Kanzawa,¹³ that SWD may overwinter as a 'winter morph'. Next, the authors elaborated on the distinctive morphology between these so called 'Winter Morph – WM' vs. 'Summer Morph – SM', highlighting the

darker coloration and increased wing size of the former. Additionally, the authors¹² reported the successful laboratory rearing of both morphs. The next year, Shearer *et al.*¹⁴ presented data suggesting a seasonal transition between two distinct 'morphs', resulting from fly trapping in Hood River (USA) throughout 2011 and 2012. The proportion of dark WM increased as the seasons progressed until December when only WM were found. This study also revealed that lab conditions (10 °C and 12:12 L:D) could induce the WM, which was characterized by dark coloration and increased cold tolerance. Photos were included to visually depict the two distinct morphotypes, suggesting a clear biphenism.¹⁴ After these early investigations, all subsequent publications on seasonal and thermal ecology of SWD acknowledged such a seasonal biphenism giving rise to such a phenotypic dichotomy: the SM vs. the WM, as reviewed in.^{1,6–8}

The expression of different phenotypes from a single genotype in response to different environments, known as phenotypic

* Correspondence to: H Colinet, Université de Rennes, CNRS, ECOBIO (Ecosystèmes, biodiversité, évolution) – UMR 6553, 35000 Rennes, France. E-mail: herve.colinet@univ-rennes.fr

Université de Rennes, CNRS, ECOBIO (Ecosystèmes, biodiversité, évolution) – UMR 6553, Rennes, France

plasticity, results in traits that can be distributed either qualitatively (i.e., polyphenism) or quantitatively (i.e., reaction norm). Hence, polyphenism typically describes a plastic response where individuals of the same genotype can develop into clearly different forms or morphs.^{15,16} Polyphenism may occur in different ways. First, from a switch in developmental pathways that produces distinct phenotypes. Endocrine mechanisms underlie such polyphenic changes. A typical example includes the expression of diapause triggered by hormonal changes in response to photoperiodic cues.^{17,18} The other possible way can occur when developing individuals encounter markedly different environments. This scenario is common in bivoltine insects, where each generation develops in different seasons, resulting in the expression of distinct traits or morphs.¹⁷

Winter captures of SWD in different regions revealed the presence of dark flies lacking mature eggs, leading the authors to suggest the occurrence of a true reproductive diapause.^{19,20} This discovery strengthened the idea that SWD may display a genuine biphenism, evident in both morphology and the diapause transition, as explicitly evoked in numerous studies.^{6,14,21–24} Because all phenotypic changes (cold tolerance, morphology, color and ovarian maturation) appeared to be solely regulated by temperature not photoperiod, Toxopeus *et al.*²⁵ expressed skepticism regarding the existence of a true diapause in SWD. Several studies confirmed that temperature was the main driver of the expression of dark WM and reproductive arrest.^{9,26} The existence of genuine biphenism is questionable. Indeed, as proposed by Nijhout¹⁷ most polyphenisms are in reality reaction norms, although this is not always easy to detect in nature. For instance, in the butterfly *Araschnia levana*, different generations develop in discretely different environments resulting in a purely biphenic polyphenism. But many intermediate forms can be produced by timed ecdysone injections or by intermediate environments indicating that a reaction norm in fact underlies this apparent polyphenism.¹⁷

In SWD, field captures consistently reported a seasonal shift in the occurrence of two morphs, associating the diapausing dark morph with winter.^{14,27} However, relying solely on body color to differentiate WM versus SW proved challenging when studies started to report intermediate phenotypes,^{20,26–28} suggesting a reaction norm rather than a true biphenism. In fact, Stockton *et al.*²⁹ tested four developmental temperatures (10, 15, 20, 25 °C) and reported the expected negative relationship between pigmentation and temperature. This study was the first to suggest that morphotype expression in SWD may represent a continuum of phenotypes in response to temperature. Clearly, the process leading to different morphotypes in SWD seemed more ambiguous than previously thought. The classification into this phenotypic dichotomy (diapausing dark WM vs. reproductive light SM) became more perplexing when findings revealed that: (i) field captured SWD did contain mature eggs during fall and winter,³⁰ (ii) WM could in fact lay a few eggs even at 10 °C²⁶, (iii) WM could quickly resume egg-laying when returned to a permissive temperature^{26,31} and (iv) SM adults could also overwinter in some regions.³² These observations challenge the idea that the dark WM is the exclusive phenotype capable of surviving winter through genuine reproductive diapause. Here, we made the general hypothesis that, in line with Nijhout,¹⁷ the presumed biphenism of SWD is nothing more than two extreme/distinct phenotypes resulting from continuous thermal reaction norms.

Past studies on the effect of developmental temperatures on insect performance typically utilized only few, often two, distinct temperatures.³³ The limited temperature range complicates the

accurate estimation of thermal optimum and limits. Additionally, this approach assumes linearity in thermal reaction norms, which often exhibit curved patterns.^{33,34} To depict continuous thermal reaction norms and clarify their shapes, it is crucial to evaluate the entire developmental thermal range. Here, we studied the characteristic traits of WM, including development, morphology, cold tolerance, metabolism, and oogenesis, across a gradient of 12 different developmental temperatures. We predicted that gradually increasing the temperature in 2 °C increments would induce continuous changes in all these traits, evidencing classical thermal reaction norms. We predicted that rearing at low temperatures would result in flies showing all the characteristic traits of the WM: extended development, dark color, larger size, high cold tolerance, reduced metabolism, and slowed oogenesis.

2 MATERIAL AND METHODS

2.1 Origin of fruit flies and breeding

The flies used in the experiments were initially captured in 2017 in a raspberry orchard in Thorigné-Fouillard, France (48° 3'41.76" N – 1°14'19.32" E). This wild strain was collected from 1500 infested raspberries. The lab stock was subsequently cultured in bottles containing standard food (per 1 L of water: 15 g of agar, 50 g of sugar, 30 g of brewer's yeast, 20 g of corn flour, 50 g of powdered carrot, 16 mL of Nipagin). The bottles were placed in incubators (Panasonic, model MIR-154) at 25 °C with a photoperiod of 12L:12D and a relative humidity of 65%.

2.2 Experimental design

To determine typical reaction norms, mature females were allowed to lay eggs for 12 h on standard food. Pools of 50 eggs were collected and placed in bottles with 15 mL of food for development at temperatures ranging from 8 to 30 °C with 2 °C increments (12 temperatures in total). The bottles with eggs were randomly distributed in the different conditions. We selected 8 °C and 30 °C as extreme temperatures to encompass the entire thermal range of SWD.^{7,10} The photoperiod was the same in all conditions (12L:12D). The bottles were monitored twice a day to collect the emerging individuals. The emergents were transferred to new food bottles and returned to their original thermal conditions to monitor various phenotypic traits. We used 20–30 bottles per temperature condition (incubator), each with 50 eggs on food, to ensure a sufficient number of emerging flies for comprehensive phenotyping. We allocated more bottles to extreme temperatures (both heat and cold), because of the predicted lower viability.

2.3 Development time and viability

For each temperature, six bottles, each containing 50 eggs, were used to assess time to emergence and viability ($n = 300$). The bottles were observed twice a day (morning and evening) to collect the emerging individuals, which were visually sexed without CO₂. The development time was determined as time from egg deposition to emergence. Viability (i.e., egg-to-adult survival) was calculated as the ratio of the number of emergents to the number of eggs laid per bottle.

2.4 Morphological traits

Morphology assessments were conducted on 7-day-old adults, with 10 females randomly taken from rearing bottles used for each temperature ($n = 10$). The females were dissected under a stereomicroscope and photos were taken using a connected camera (AxioCam ERc 5 s, Carl Zeiss Microscopy GmbH, Germany). The

size of the ovipositor (left side) was measured (Fig. 2A). On the left wing, we measured the length of the fourth longitudinal vein and the surface of the intervein region I4 (Fig. 2B). Additionally, we measured the surface and a pigmentation level of each of the five abdominal segments (S1–S5; see in Fig. 3). All photos were processed using ZEN software (Carl Zeiss Microscopy GmbH, Germany). Segment brightness was determined using the ZEN software, which evaluated the grey coloration of each pixel on a 255-level scale, ranging from white (255) to black (0). The brightness index for each segment was then calculated by dividing the obtained value by the segment area.

2.5 Cold tolerance

Females assessed for cold tolerance were all tested at 7-d-old. Two metrics were employed: acute cold survival and critical thermal minimum (Ctmin). For the first, 10 glass tubes, each containing 10 females ($n = 10 \times 10$) randomly taken from rearing bottles, were immersed in a cryostat bath (Lauda ECO RE 630S, Germany) set at $-5\text{ }^{\circ}\text{C}$ for 100 min. Subsequently, the flies were returned to food vials by groups of 10 at their developmental temperatures, and survival was scored after 48 h. For Ctmin, we used a vertical double-walled glass knock-down column, connected to a cryostat (Lauda ECO RE 630S, Germany). About 100 females ($n = 100$) randomly taken from rearing bottles were placed in the upper part of the column after which the temperature was gradually decreased from 20 to $-5\text{ }^{\circ}\text{C}$ at a rate of $0.5\text{ }^{\circ}\text{C}/\text{min}$. The temperature inside the column was monitored using a thermocouple (type K) connected to a thermometer (C8600, Comark Instruments, UK). Ctmin values were individually recorded at the point when flies entered chill coma, and consequently fell out of the column.

2.6 Respirometry

For metabolic rate we used both males and females of 24 h post-emergence. We opted to test the adults shortly after emergence to minimize any differences in physiological age across various thermal conditions. For each sex and developmental temperature (except for $8\text{ }^{\circ}\text{C}$), 40 individuals randomly selected from the development bottles were tested ($n = 40$). One hour before each test, flies were placed at $20\text{ }^{\circ}\text{C}$ to acclimatize before testing. The individual CO_2 production of each fly was measured using a multi-channel (16-chamber) flow-through MAVEn respirometry system (Sable Systems International, Las Vegas, USA) at $20\text{ }^{\circ}\text{C} \pm 0.5$. The system comprised a differential CO_2 analyzer (Li-Cor7000) and a 16-channel flow management and signal processing system. The MAVEn was calibrated before experiments using pure nitrogen and CO_2 at 10 ppm. To prevent flies from drying out during measurements, the pumped air passed through a humidifier (Nafion bottle, Sable Systems International) before entering the chamber. The flow rate in each chamber was set at 20 mL min^{-1} . Flies were placed without anesthesia in individual chambers, each consisting of a transparent plastic tube (46 mm long and 6 mm in diameter). The MAVEn system was configured to measure each chamber for 120 s (Dwell Time) with baseline measurements (for 120 s) taken every eight chambers (Interleave ratio). For each chamber, three cycles of measurements were taken, resulting in three repeated measurements per chamber. After the test, each fly was weighed using a microbalance (precision $1\text{ }\mu\text{g}$) (Mettler Toledo XP2U, Columbus, USA). All data transformations and extractions were conducted in ExpeData Software (Sable Systems International, Las Vegas, USA), utilizing macros provided by Sable Systems for the transformation of raw data. After extracting the

data for each chamber, the mass-corrected metabolic rate was calculated as the ratio of CO_2 produced (mL/min) to body weight (mg) of the fly. The standard metabolic rate (SMR) was obtained by selecting the minimum rate obtained from three cycles.

2.7 Dynamics of egg maturation

At each developmental temperature, newly emerged females randomly taken from rearing bottles were grouped in pools of 15 in new food vials, with multiple vials/pools for each temperature, and then returned to their respective incubators. Subsequently, at each time point, a pool of 15 females was dissected under stereomicroscope to examine egg maturation stage in ovaries. Dissections were conducted at various intervals depending on temperature: every 5 days for 10 and $12\text{ }^{\circ}\text{C}$, every 2 days for $14\text{ }^{\circ}\text{C}$, daily for 16 and $18\text{ }^{\circ}\text{C}$, and twice daily (morning and evening) for the first 2 days followed by daily dissections for $20\text{ }^{\circ}\text{C}$ or higher. An egg was considered as mature when vitellogenesis was complete, appendage visible, and chorion formed, corresponding to stages 13–14 (see photos c,d in Fig. 7A).³⁵ For each temperature and time, the percentage of females with at least one mature egg was obtained based on the 15 dissected females at each time point.

2.8 Statistical analyses

All statistical analyses were conducted in R-studio (Version 2022.07.2 Build 576) using R version 4.2.2. Viability data were analyzed using binomial GLM with a logit link function for binary outcome (dead vs. alive). To model the thermal reaction norm for viability and estimate parameters, we used 'rTPC' and 'nls.multstart' packages.³⁶ We selected the best nonlinear equation according to the lowest AIC, the accuracy of parameters relative to observations, and the fit of data. Development rate ($1/\text{development time}$) according to temperature and sex was analyzed using GLM with an inverse Gamma link function. We also fitted a quadratic term (order 2) for temperature, and model comparison (linear vs. quadratic) was conducted based on the Akaike Information Criterion (AIC) using the 'MuMIn' package. To model the reaction norm for development rate and estimate key parameters, we used the 'devRate' package.³⁷ We tested both the classical linear equation and six non-linear equations, selecting the best model based on the lowest RMSE, accuracy in estimating parameters, and overall data fit. For Ctmin and morphological traits, GLMs were used as a function of developmental temperature, comparing linear vs. quadratic models using AIC criteria. For acute cold tolerance, we used a binomial GLM according to temperature with a logit link function for binary outcome (dead vs. alive). The SMR was analyzed with GLM considering temperature, sex and the interaction, and we also compared linear vs. quadratic model. Thermal performance curves for SMR were also modelled using 'rTPC' and 'nls.multstart' packages. Ovarian maturation was first analyzed using a binomial GLM with a logit link function for binary outcome (mature vs. immature) according to developmental temperature, time and interaction. Subsequently, we analyzed the temporal dynamics of the percentage of egg maturation at each temperature independently. At high temperatures, we observed steady and rapid increases over time, whereas at low temperatures, initial lags were followed by a sharp upward trend. Consequently, we conducted analyses using both linear and nonlinear models, by fitting an exponential curve *via* the 'nls' function. We compared two models based on AIC criteria. Finally, regarding the WM presumed to be a morphologically distinct variant, we conducted

bimodality tests on all morphology traits using Hartigan Dip tests in 'diptest' package.

3 RESULTS

Not all measurements were feasible for all developmental temperatures, particularly at extreme cold and heat. As can be seen in Fig. 1A, viability at 8 and 30 °C were very low (2.3% and 5.6%, respectively). Consequently, the emergent stock was too limited to conduct all planned measurements at these two temperatures. Only morphological measures were performed on flies at 8 °C. For individuals developed at 30 °C, there was also a high early mortality (most emergents died within 48 h). Consequently, only SMR could be assessed on flies of 1 day of age at 30 °C.

3.1 Viability and development time

Figure 1A depicts the percentage of viability that gradually increased from the minimum (8 °C) to an optimum estimated around 22–24 °C, depending on models, followed by a rapid decline to nearly zero at 30 °C. The GLM showed that viability varied with developmental temperature ($\chi^2 = 1199.4$, $P < 0.001$). Among all the non-linear equations tested, the 'kamykowski' model proved to be the most suitable, demonstrating the best fit and coherent estimated parameters (see Table S1). This model estimated T_{min} , T_{max} , and optimal temperature (Top) at 7.66, 30.27, and 21.87 °C, respectively. The prediction curves from all the other models are shown in Figure S1.

For development rate, the quadratic model (qm) better captured the data compared to the linear model (lm) (AICqm = -11 897.2 vs. AIClm = -9740.6). Development rate (Fig. 1B,C) was significantly influenced by temperature ($\chi^2 = 14 323.4$, $P < 0.001$) and sex ($\chi^2 = 28.6$, $P < 0.001$), with no significant interaction ($\chi^2 = 3.8$, $P = 0.152$). On average, development was slightly faster in males. Notably, at low temperatures, the development time was particularly long, lasting about 3 and 2 months at respectively 8 and 10 °C (Fig. 1B,C). Among the tested linear and non-linear equations, the 'perf2' model was the most suitable, displaying the best fit and coherent estimated parameters (see Table S2). This non-asymptotic model successfully estimated both lower and upper limits for development at respectively 7.9 and

31.3 °C for males and 7.5 and 31.8 °C for females. The prediction curves resulting from the other models are presented in Fig. S2.

3.2 Morphological traits on the ovipositor and wings

All metrics indicated a gradual reduction in body size with increasing temperatures (Fig. 2C–E). None of the morphological traits showed significant bimodality (Dip tests, $P > 0.05$). The quadratic models better described the data on morphological traits compared to the linear models for all metrics (Table 1). The GLMs indicated that developmental temperature had a significant effect on the size of the ovipositor, wing length, and wing cell area (Table 1).

3.3 Morphological traits on the abdomen

The panel of images in Fig. 3 demonstrates a continuum of morphotypes associated with abdomen pigmentation in response to the developmental temperatures. None of the morphological traits showed significant bimodality (Dip tests, $P > 0.05$). For all traits on the abdomen, the quadratic models better described the data compared to the linear models (Table 1). Across all metrics, lower temperatures correlated with larger size and darker pigmentation, both of which gradually diminished as temperature increased (Figs 3 and 4). GLMs highlighted a significant impact of developmental temperature ($P < 0.001$) for all segment areas and their pigmentation (i.e., the brightness index) (Table 1).

3.4 Cold tolerance

The C_{tmin} gradually decreased as the developmental temperature declined (Fig. 5A). The quadratic model better captured the C_{tmin} data compared to the linear model (AICqm = 3343.1 vs. AIClm = 3403.4). C_{tmin} was significantly influenced by developmental temperature ($\chi^2 = 462.29$, $P < 0.001$). The developmental temperature also had a significant effect on the acute cold survival ($\chi^2 = 731.62$, $P < 0.001$). Females raised at low temperatures (10–16 °C) were highly cold-tolerant, while those at higher temperatures (26–28 °C) had poor cold survival, with intermediate cold tolerance observed for temperatures in between (Fig. 5B).

3.5 Standard metabolic rate (SMR)

For the SMR, the quadratic model better fit the data compared to the linear model (AICqm = -20 146.7 vs. AIClm = -20 004.7). The

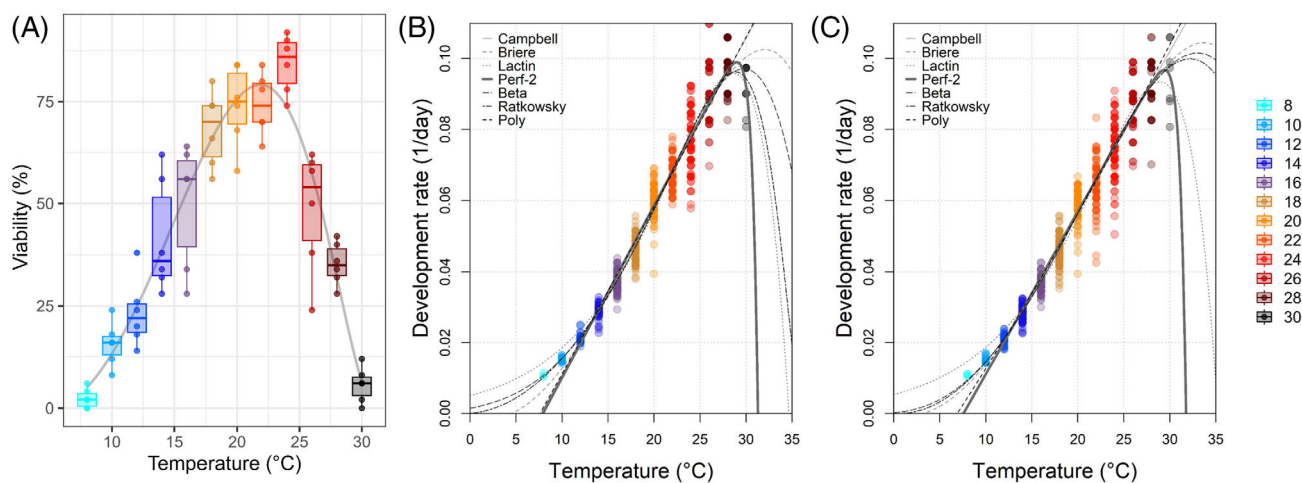


Figure 1. (A) Percentage of viability, from egg to adult, according to developmental temperature (8 to 30 °C). The grey curve depicts the predictions from the Kamykowski model. (B) & (C) Development rate, from egg to adult, according to developmental temperature (8 to 30 °C) in males (B) and females (C). The curves depict the predictions from the different models indicated within the plots.

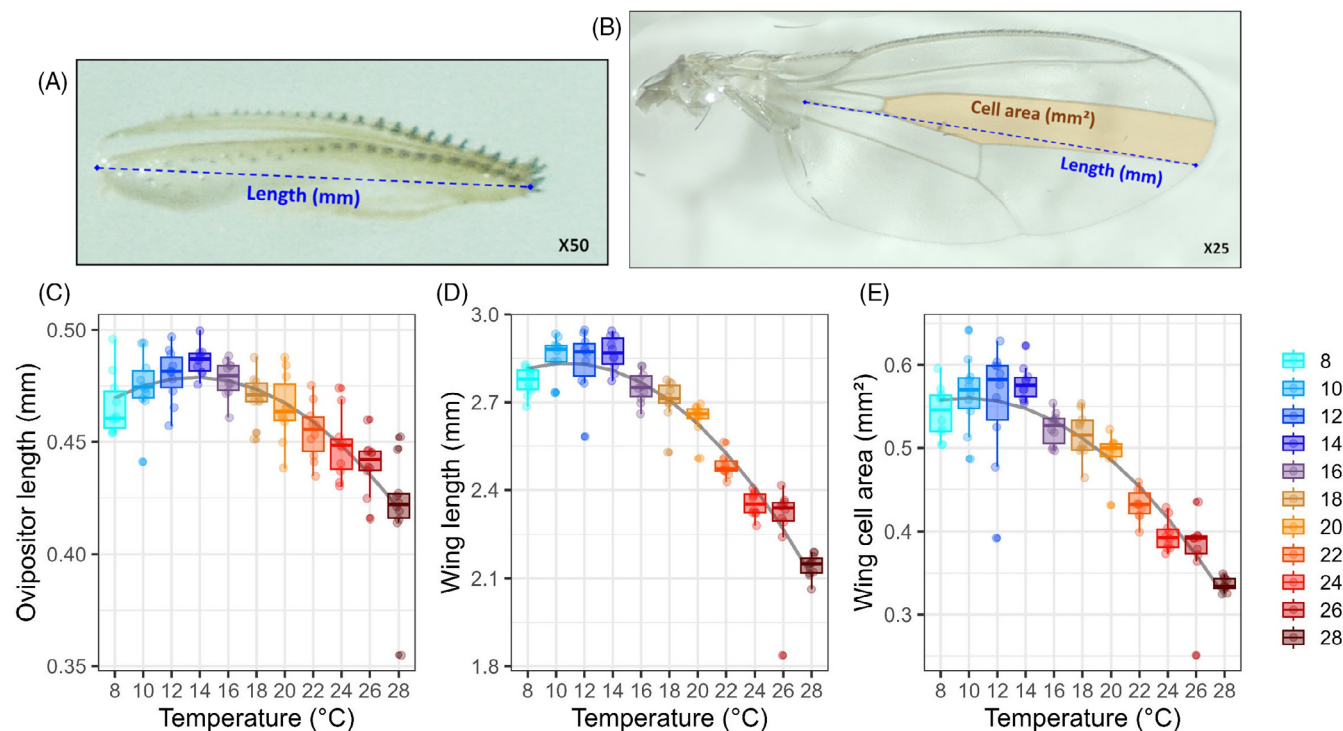


Figure 2. Illustrations of the morphometric measurements made on the ovipositor (A) and on the wing (B). Size of the ovipositor (C), length of the fourth longitudinal vein of the wing (D), and area of the interveinal region I4 (E) as a function of developmental temperature (8 to 28 °C). The grey curves represent the predictions of the quadratic models.

Table 1. Statistical analysis of linear and polynomial models describing temperature-dependent changes in morphometric parameters: ovipositor length, wing length, segments area, and brightness. Comparative analysis of models with AIC evaluation. A positive Δ AIC suggests that the polynomial model is better to describe the data

Variable	Linear model				Polynomial model				Δ AIC
	DF	F-value	P-value	AIC	DF	F-value	P-value	AIC	
Ovipositor Length	1	89.34	<0.001	-575.48	2	95.83	<0.001	-620.09	44.61
Wing Length	1	386.43	<0.001	-151.80	2	474.29	<0.001	-234.25	82.45
Wing Area	1	306.18	<0.001	-367.85	2	252.57	<0.001	-409.85	42.00
Segment 1 Area	1	57.94	<0.001	-287.80	2	83.67	<0.001	-342.12	54.32
Segment 1 Brightness	1	91.69	<0.001	1327.33	2	104.08	<0.001	1278.11	49.22
Segment 2 Area	1	48.81	<0.001	-336.13	2	31.21	<0.001	-343.65	7.52
Segment 2 Brightness	1	80.41	<0.001	1298.16	2	59.84	<0.001	1278.80	19.36
Segment 3 Area	1	47.78	<0.001	-306.89	2	27.12	<0.001	-309.70	2.81
Segment 3 Brightness	1	102.20	<0.001	1336.67	2	68.32	<0.001	1321.41	15.26
Segment 4 Area	1	69.37	<0.001	-341.20	2	35.99	<0.001	-341.22	0.02
Segment 4 Brightness	1	178.32	<0.001	1378.71	2	118.27	<0.001	1359.65	19.06
Segment 5 Area	1	73.41	<0.001	-472.06	2	36.60	<0.001	-470.34	-1.71
Segment 5 Brightness	1	153.04	<0.001	1548.48	2	96.32	<0.001	1534.29	14.19

SMR (Fig. 6) was significantly influenced by temperature ($\chi^2 = 532.52, P < 0.001$) and sex ($\chi^2 = 10.35, P = 0.0012$), with a sex-by-temperature interaction ($\chi^2 = 11.73, P = 0.0028$). On average, SMR was slightly higher in males. SMR values peaked around 24 °C (optimal), depending on models, with a decline at lower and higher developmental temperatures (Fig. 6). Among all tested models, the ‘Rezende’ model emerged as the most suitable, displaying the best fit and coherent estimated parameters (see Table S3). This model estimated the optimal temperature (Topt)

and critical thermal maximum (Tmax) at 24.68 and 37.27 °C respectively. The prediction curves resulting from the other models are presented in Figure S3.

3.6 Dynamics of egg maturation

Regarding the percentage of females with mature eggs, the model revealed significant impacts of temperature ($F = 258.78, P < 0.001$), time ($F = 109.87, P < 0.001$) and a significant interaction ($F = 292.55, P < 0.001$). The interaction indicated that, at

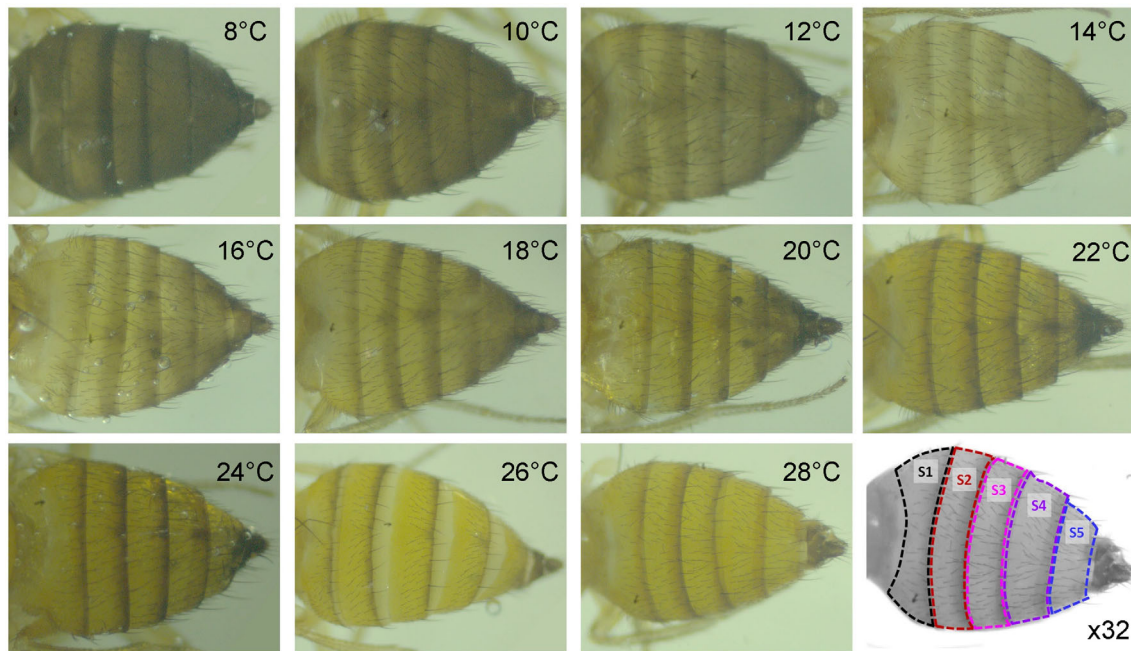


Figure 3. Panel of images showing a continuum of morphotypes in which abdomen pigmentation decreases in response to increasing developmental temperature (indicated in the top right of each image). The last picture, on the bottom right, illustrates the five segments (S1–S5) used to determine area and brightness.

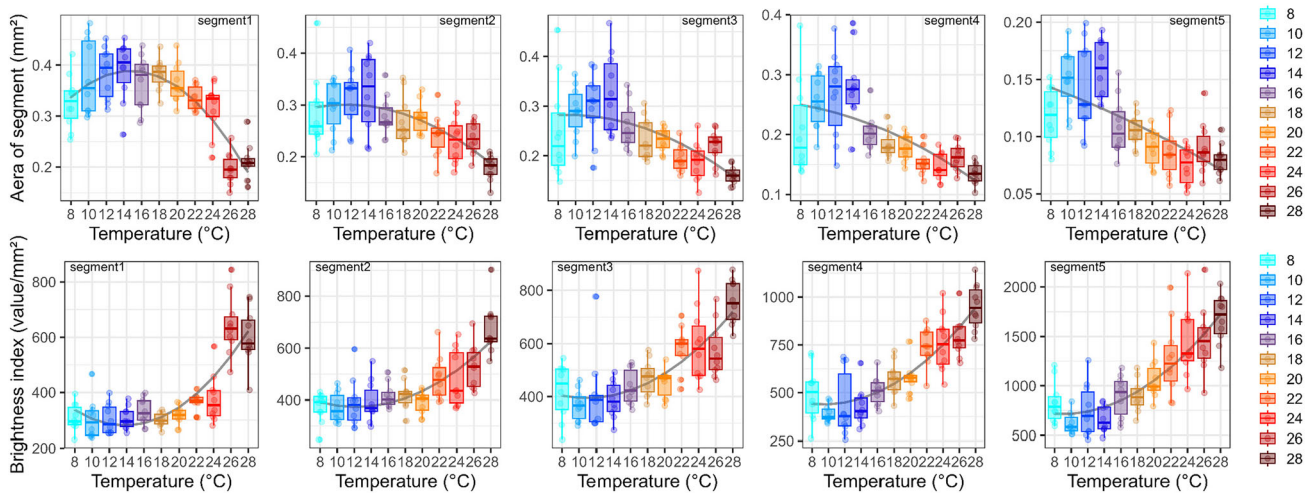


Figure 4. Area of abdominal segments S1 to S5 (top of panel) and brightness index of each segment S1 to S5 (bottom of panel) as a function of developmental temperature (8 to 28 °C). The grey curves represent the predictions of the quadratic models.

lower temperatures, the temporal dynamics of egg maturation occur more slowly than at higher temperatures, though it was not completely halted (Fig. 7B). At each developmental temperature, the percentage of ovarian maturity over time was modelled and the results are summarized in Table 2. Non-linear (exponential) models better captured the data at low temperatures (10–14 °C), while linear models effectively depicted the steady increase in egg maturation rate at 16 °C and above (Table 2). Based on these models, we estimated the time required to reach 50% of females with mature eggs, for each temperature. As illustrated in Fig. 7C, the time to 50% mature females increased with decreasing temperature: approximately 2–3 days for temperatures from 28 to 20 °C,

around 8–10 days for 18–16 °C, and several weeks at lower temperatures.

4 DISCUSSION

The overwintering strategy of SWD has been supposed to rely on a clearly distinct phenotype: the WM, which is dark and diapausing, as opposed to the SM, which is light and active in terms of reproduction.^{1,3,6–8,12,14} However, the existence of a genuine biphenism in SWD appeared more ambiguous than previously believed and this question needed to be solved. In this study, we made the prediction that, in line with Nijhout,¹⁷ the presumed

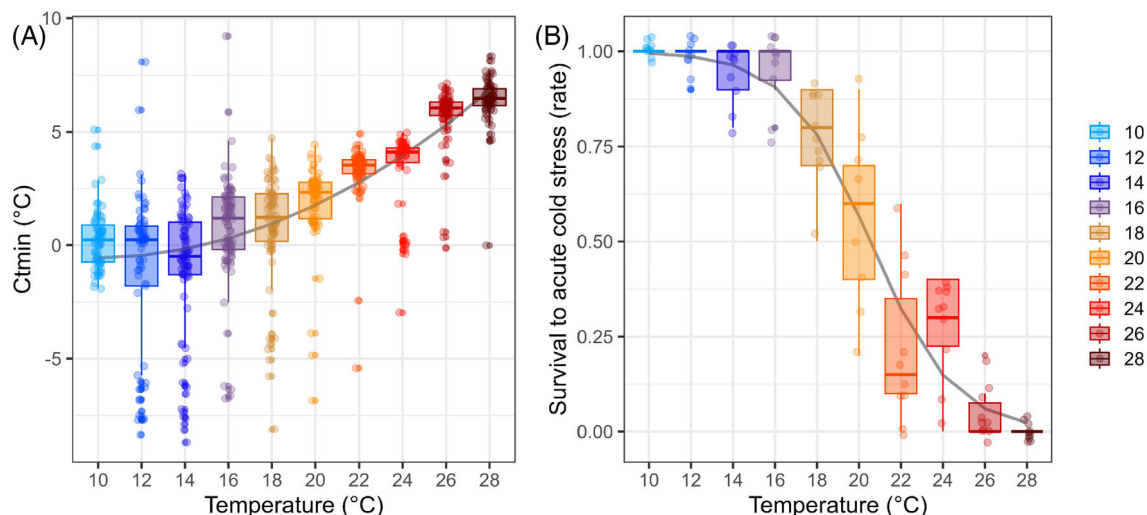


Figure 5. Cold tolerance as a function of developmental temperature (10 to 28 °C). (A) Minimum critical temperature (Ctmin) and (B) survival measured 48 h after exposure to acute cold stress (−5 °C for 100 min). The grey curves represent the predictions of the GLM models.

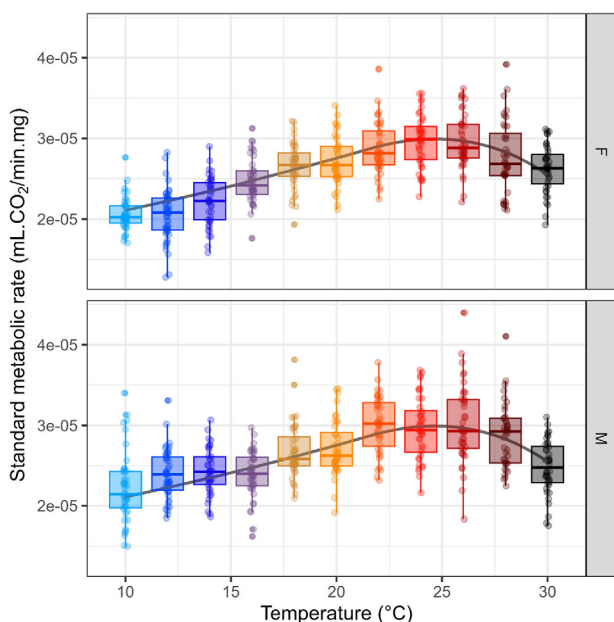


Figure 6. Standard metabolic rate (SMR) as a function of developmental temperature (10 to 30 °C) in females (top) and males (bottom). The grey curves depict the predictions from the Rezende model.

biphenism of SWD could represent nothing more than the expression of two ‘extreme’ phenotypes resulting from continuous thermal reaction norms. We used a gradient of 12 different developmental temperatures to highlight continuous reaction norms in the measured traits. Many phenotypes are not single traits or sets of traits but curves traits that are functions of some continuous variable. Developmental reaction norms, performance curves or growth curves are classical examples of curves traits that can have a variety of different shapes.³³

The viability exhibited the typical shape of a thermal performance curve³⁸ which delineates the range temperatures in which performances can be maintained. Viability increased progressively from the critical minimum (7.66 °C) to an optimum

estimated near 21.87 °C, then decreased very rapidly to reach a critical maximum at 30.27 °C. These values fit with earlier observations at constant temperatures.^{7,39} Here, we tested a number of models to describe the continuous relationship between viability and developmental temperature because the choice of model can affect the estimation of descriptors.⁴⁰ Yet, most of the models estimated fairly similar values, apart from the asymptotic models close to zero, which gave estimates with little ecological relevance.

As for viability, we observed a continuous reaction norm for growth rate according to temperature. The non-asymptotic models successfully estimated lower and upper limits for development at around 7.5 and 31 °C. These values are pretty similar to those describing the limits for viability, suggesting strong physiological constraints above and below these limits. These values also fit well with previous observations.⁷ Notably, low developmental temperatures (<14 °C) led to an extended time to emergence (2–3 months). So, in the wild, if certain individuals begin their development at the end of the season in protected microhabitats (soil or litter) and manage to develop to adult, as has been shown,^{10,30} then these individuals would emerge in the middle of winter with all the characteristics of the WM.

Most *Drosophila* species exhibit continuous thermal reaction norms for wing size and morphology.^{41,42} Without surprise, we found a continuous reaction norm in SWD as well. As shown by Fraimout *et al.*⁴³ using only three different temperatures, development at low temperature leads to larger bodies and wings. Continuous non-linear thermal reaction norms were also observed for the pigmentation of the abdomen of SWD, consistent with other *Drosophila* species.^{44,45} These response curves were continuous as expected and effectively described by quadratic polynomials as previously found in other species.^{44,45} Additionally, we observed a continuous thermal reaction norm in the size of the ovipositor which aligns with a former study on SWD that used two temperatures.⁴⁶ The visual representation of the data clearly demonstrates that all traits exhibit continuous variation with temperature, with many intermediate phenotypes, showing no clear evidence of bimodality. Regarding the WM presumed to be a morphologically distinct variant, we conducted bimodality tests on morphology traits, and none showed

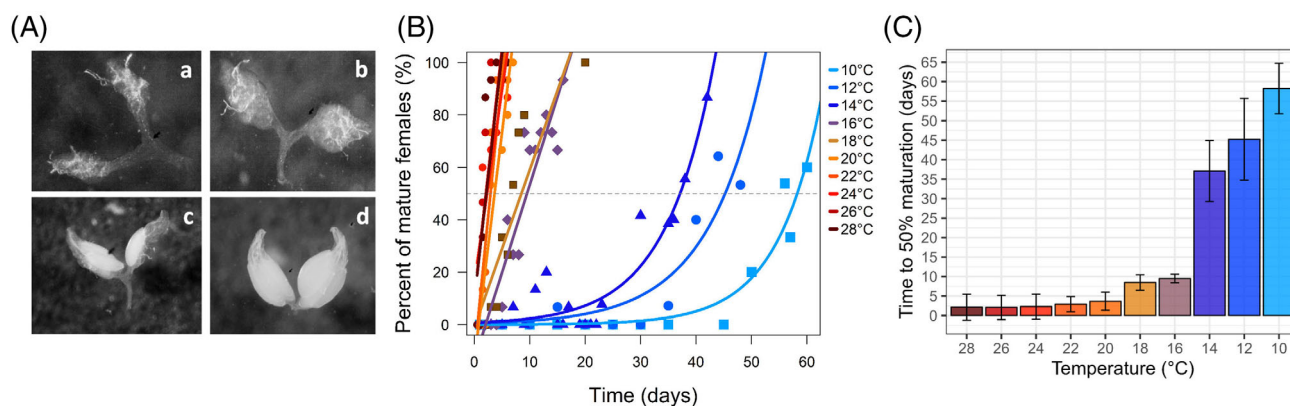


Figure 7. (A) Panel of images showing various stages of maturation during oogenesis. An egg was considered as mature when vitellogenesis was complete, appendage visible, and chorion formed (i.e., stages 13–14), corresponding to the two bottom images (C & D). (B) Temporal dynamics of egg maturation as a function of developmental temperature (10 to 28 °C). The lines or curves depict the predictions from the models. The dashed horizontal line indicates 50% of females with mature eggs. (C) Estimated time required to reach 50% of females with mature eggs (\pm SE) as a function of developmental temperature (10 to 28 °C).

Table 2. Statistical outputs from modeling temperature-dependent effects on temporal dynamics of egg maturation: comparative analysis of linear and non-linear (exponential) models with AIC evaluation. A positive Δ AIC suggests that the exponential model is better to describe the data

Temperature	Linear model						Non-linear (exponential) model						Δ AIC
	AIC	Term	Estimate	SE	t-value	P-value	AIC	Term	Estimate	SE	t-value	P-value	
10	113.1	Intercept	-13.17	7.569	-1.740	0.110	91.9	a	0.033	0.049	0.680	0.511	21.2
		Time	0.827	0.203	4.069	0.002		b	0.126	0.025	4.946	0.000	
12	99.4	Intercept	-14.25	9.185	-1.551	0.155	90.1	a	0.553	0.653	0.847	0.419	9.30
		Time	1.202	0.315	3.814	0.004		b	0.100	0.026	3.814	0.004	
14	169.4	Intercept	-15.44	6.259	-2.467	0.024	144.5	a	0.751	0.408	1.842	0.082	24.90
		Time	1.633	0.279	5.854	0.000		b	0.113	0.014	8.112	0.000	
16	133	Intercept	-13.67	6.737	-2.029	0.062	143.2	a	12.69	4.389	2.892	0.012	-10.20
		Time	6.706	0.697	9.625	0.000		b	0.129	0.026	4.952	0.000	
18	93.8	Intercept	-1.206	9.108	-0.132	0.898	100.1	a	20.47	7.464	2.743	0.025	-6.30
		Time	6.03	1.100	5.481	0.001		b	0.084	0.023	3.591	0.007	
20	88.7	Intercept	-13.59	12.69	-1.071	0.320	93.6	a	14.57	8.740	1.667	0.140	-4.90
		Time	17.41	3.189	5.459	0.001		b	0.291	0.101	2.879	0.024	
22	77.3	Intercept	-12.01	10.82	-1.110	0.309	84.3	a	17.75	9.158	1.938	0.101	-7.00
		Time	21.28	3.164	6.726	0.001		b	0.313	0.102	3.060	0.022	
24	84.5	Intercept	16.15	16.955	0.952	0.378	86.3	a	31.42	13.30	2.362	0.056	-1.80
		Time	14.67	4.959	2.959	0.025		b	0.197	0.093	2.127	0.078	
26	84	Intercept	10.21	16.48	0.620	0.558	87.4	a	32.67	13.67	2.389	0.054	-3.40
		Time	19.06	4.819	3.954	0.008		b	0.218	0.089	2.439	0.051	
28	85.1	Intercept	9.422	17.55	0.537	0.611	88	a	31.62	14.15	2.235	0.067	-2.90
		Time	18.75	5.134	3.652	0.011		b	0.220	0.095	2.306	0.061	

significant effect. Altogether, these data demonstrate that gradually increasing the developmental temperature in 2 °C increments induced continuous changes in all these morphological traits, evidencing classical non-linear thermal reaction norms in SWD.

The late-season emergence of SWD flies of bigger size, following an extended developmental period, could potentially be advantageous. Such an augmentation in size may facilitate increased storage of energy resources, including fat and cryoprotective compounds such as sugars, thereby contributing to enhance cold

tolerance.^{47,48} Furthermore, larger wings in SWD could enhance flight capabilities as suggested by Fraimout *et al.*⁴³ Studies on *D. melanogaster* have indicated that flies reared and acclimated at cold have improved flying ability at lower temperatures due to increased wing area and length.^{49,50} Consequently, larger wings may assist winter SWD in flying under cold conditions. The recurrent captures of larger and darker flies in winter traps underscores their ability to fly even in cold weather. Furthermore, there is substantial support for the hypothesis that increased melanism at low temperatures could confer some fitness advantages

such as thermoregulation in line with the thermal melanism theory, camouflage against predators, or improving pathogen resistance due to melanin's involvement in immune response.⁵¹

Our data showed gradual nonlinear changes in cold tolerance according to temperature. Flies raised at lower temperatures demonstrated better cold tolerance than those at higher temperatures, with intermediate phenotypes in between. The WM, generally obtained by developing flies at temperatures between 10 and 15 °C, was reported to exhibit improved cold tolerance compared to SM.^{12,14,29,31,52} The observed gradual variations in cold tolerance in response to developmental temperatures result from thermal acclimation. Various types of acclimation exist, including developmental acclimation, gradual acclimation, and rapid acclimation (also denoted rapid hardening).⁵³ In our experiments, individuals underwent both developmental and gradual acclimation as they progressed through development and were then maintained in the adult stage at various low temperatures before testing. These acclimation treatments and their combinations are well-known to promote cold tolerance in *Drosophila*.^{48,53,54} Our data support the findings of Schou *et al.*⁵⁵ on the responses of *D. melanogaster* to 10 developmental acclimation conditions. They observed a gradual 8 °C decrease in C_{min} with lower temperatures, mirroring our findings. Our results highlight once again the existence of a continuous range of phenotypes. This aspect went unnoticed in SWD, likely due to previous cold tolerance studies focusing solely on the extreme/distinct phenotypes (SM vs. WM) of the reaction norms without examining the intermediates. Yet, our study shows that these intermediaries do exist.

Metabolic depression is a universal aspect of diapause.⁵⁶ So far, no study has attempted to determine whether there is such a depression in the WM supposed to be in reproductive diapause. Metabolic depression varies with diapause strategies, being more pronounced in immobile stages, while diapausing free-living and mobile stages generally experience milder depression.¹⁸ We found that lowering developmental temperature in 2 °C increments led to a gradual reduction in SMR, illustrating again classic thermal reaction norm. Our findings suggest a continuous, temperature-dependent adjustment of metabolic rate rather than a pronounced diapause-related metabolic depression. The model predicted optimal temperature and critical thermal maximum for SMR at 24.6 and 37.2 °C, respectively. Our values and the shape of the reaction norm align with observations in *D. melanogaster*.⁴⁰ The decline in SMR at high temperatures likely resulted from stress due to supra-optimal developmental conditions, as indicated by reduced viability and early adult mortality.

Regarding the ovarian maturity, we found that at low temperatures the temporal dynamics of eggs maturation was slowed but not arrested. Oogenesis slowed significantly at 14 °C and below, the temperatures typically used to induce WM. Notably, at 10, 12, and 14 °C, females exhibited a delay before showing mature eggs after approximately 50, 35, and 30 days, respectively. So, under constant cold conditions, we observed spontaneous resumption of ovarian development that occurred faster when temperature increased. This is not in line with the characteristics of diapause which requires specific diapause-terminating conditions to end.¹⁸

Reproductive diapause and quiescence are distinct forms of dormancy used to suspend or delay reproduction. While diapause tends to be prolonged and seasonally (hormonally) regulated, mainly by photoperiod, quiescence is a short-term state that does not follow seasonal patterns.¹⁸ Another key distinction is the

reversibility. Diapause requires specific conditions to terminate, while quiescence ceases as soon as permissive conditions return.^{18,57} In *D. melanogaster* reproductive diapause is recognized to be shallow⁵⁸ as it is predominantly regulated by temperature,^{59,60} termination occurs spontaneously after a few weeks,^{59,61} and egg laying resumes as soon as permissive temperatures return.^{59,62} Hence, authors proposed calling the reproductive dormancy of *D. melanogaster* quiescence instead of diapause.⁶² A similar situation seem to occur in SWD. Indeed, temperature primarily drives the transition into the dark WM and reproductive slowdown^{9,25,26} and egg-laying in phenotypes supposed to be dormant quickly resumes (2–4 days) at permissive temperature.^{26,31}

Distinguishing between reproductive diapause and quiescence is complex, also partly due to unclearly defined terminology.^{18,57} As a result, the literature on SWD continues to use both terms. Our data reveal that developmental cold acclimation generates dark and cold-tolerant females, with reproductive delays ending spontaneously end more or less quickly depending on the temperature. Following Košťál's¹⁸ guidelines such syndrome should be classified as reproductive quiescence, as it seems to lack discernible preparatory and termination phases and swiftly influenced by external conditions. To avoid any ambiguity we propose to use the term 'reproductive dormancy' which encompasses both responses.

To conclude, we showed that rearing at low temperatures resulted in SWD flies showing all the characteristic traits of the so-called 'winter morph', such as extended development, dark color, larger size, increased cold tolerance, reduced metabolism, and slowed oogenesis. Gradually increasing the developmental temperature induced gradual changes in all these traits, evidencing classical thermal reaction norms. In certain species, environmental cues can initiate a developmental switch, resulting in a phenotypic dichotomy. This may manifest as either growth or reproductive arrest, accompanied or not by distinct differences in morphology, physiology, and life history traits. Here, we found no evidence of such developmental switch or polyphenism in response to developmental conditions. We suggest that in nature, SWD might undergo a scenario akin to that observed in *Araschnia levana*, where distinct generations develop in discrete environments. This creates an apparent biphenism, which, in reality, stems from continuous reaction norms.¹⁷ In SWD, low temperatures (< 14 °C) led to extended development (2–3 months) and when adults were maintained at cold, the time to ovarian maturation required another 1–2 months. Given these time scales, which can encompass seasons, it is conceivable that SWD could emerge slowly in late autumn or even winter, as shown by Raynaud-Berton *et al.*¹⁰ and thus display all the phenotypic traits associated with the cold end of the different reaction norms. This could also lead to the expression of intermediary phenotypes as reported in SWD. We recommend avoiding the use of the term 'winter morph' as it implies the existence of a true polyphenism. Instead, we suggest using terms like 'winter-acclimated' or 'winter phenotype' to describe the dark, cold-tolerant SWD flies undergoing a shallow reproductive dormancy. Acknowledging the continuous nature of SWD responses to temperature is crucial for refining existing models and enhancing control measures against this invasive species. Traditional models of SWD biology and ecology can oversimplify the species' response to environmental cues if they assume distinct morphological or physiological states (e.g., distinct WM). By recognizing continuous thermal response norms, models can be refined to incorporate more realistic

scenarios of how SWD populations vary with different temperatures, and thus improve the accuracy of predictions regarding population dynamics, geographic distribution and seasonal dynamics.

AUTHOR CONTRIBUTIONS

A. Kustre: investigation, formal analysis, writing original draft.

H. Colinet: conceptualization, investigation, formal analysis, writing original draft, review & editing, funding acquisition, supervision.

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CONFLICT OF INTEREST DECLARATION

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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