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Drosophila suzukii

Thomas Enriquez

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Par

Thomas ENRIQUEZ

Mécanismes physiologiques sous-jacents à la plasticité de la thermotolérance chez la drosophile invasive *Drosophila suzukii*

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**Mécanismes physiologiques sous-jacents à la
plasticité de la thermotolérance chez la drosophile
invasive *Drosophila suzukii***

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*“When the air gets colder and the sun won't shine
When the winter comes he'll be a slow slow fly fly
He'll be a slow slow fly fly
He'll take a long long time time
He'll be a slow slow fly fly”*

– *The Presidents Of The United States Of America: ‘Slow Slow Fly’* –

<https://www.youtube.com/watch?v=wXwhwiuIq5o>



"There cannot be a greater mistake than that of looking superciliously upon practical applications of science. The life and soul of science is its practical application..."

Lord Kelvin - Popular Lectures Vol. I, May 3, 1883

Table des matières

Liste des publications	xi
Liste des abréviations	xii
Liste des figures	xiii
Liste des tableaux	xiii
Introduction générale	1
Avant-propos	2
I. La plasticité de la thermotolérance, un facteur déterminant du succès des invasions biologiques	3
I.1. La problématique des espèces invasives	3
I.2. La température : un facteur fondamental influençant la biologie des organismes	4
I.3. Les différentes stratégies de thermorégulation.....	5
I.4. Les courbes de performance.....	5
I.5. Les stratégies de tolérance au froid des insectes	7
I.6. Adaptation de la tolérance au froid chez les insectes.....	10
I.7. La plasticité de la tolérance au froid des insectes	10
I.7.a. Définition de la plasticité phénotypique.....	10
I.7.b. Ajustements comportementaux	12
I.7.c. L'acclimatation au froid	13
I.7.d. Les fluctuations thermiques	18
I.8. Le rôle de la plasticité de la tolérance au froid dans le succès des invasions biologiques	19
II. Mécanismes physiologiques de la plasticité de la tolérance au froid	20
II.1. Les dégâts provoqués par les températures froides	20
II.1.a. Stress froid extrême et formation de gel	20
II.1.b. Dégâts directs et indirects provoqués par un stress froid modéré	20
II.2. Mécanismes sous-jacents à l'augmentation de la tolérance au froid.....	26

II.2.a.	Maintien de la structure des macromolécules	26
II.2.b.	Maintien de l'homéostasie cellulaire.....	30
II.2.c.	L'importance des réserves énergétiques.....	31
III.	<i>Drosophila suzukii</i> , présentation de l'espèce	32
III.1.	Taxonomie et identification	32
III.2.	Aire de répartition actuelle	34
III.3.	Les paramètres du succès de son invasion	35
III.3.a.	Cycle de vie	35
III.3.b.	Hôtes et compétition interspécifique	36
III.3.c.	Faible pression de prédation dans les zones envahies	37
III.4.	La thermotolérance de <i>D. suzukii</i> favorise-t-elle son invasion ?.....	37
III.4.a.	Thermotolérance basale	37
III.4.b.	Thermotolérance induite.....	38
IV.	Objectifs et hypothèses de ma thèse.....	40
	Chapitre 1 : Thermotolérance basale de <i>D. suzukii</i>	43
	Article 1: Basal tolerance to heat and cold exposure of the spotted wing drosophila, <i>Drosophila suzukii</i>	43
	Chapitre 2 : Effet des fluctuations thermiques sur la survie et les traits d'histoire de vie de <i>Drosophila suzukii</i>	65
	Article 2: Effects of fluctuating thermal regimes on cold survival and life history traits of the spotted wing Drosophila (<i>Drosophila suzukii</i>).....	65
	Chapitre 3 - Mécanismes sous-jacents à la plasticité de la thermotolérance de <i>Drosophila suzukii</i>	86
	Article 3 : Cold Acclimation Favors Metabolic Stability in <i>Drosophila suzukii</i>	87
	Article 4: Cold acclimation triggers lipidomic and metabolic adjustments in the spotted wing drosophila <i>Drosophila suzukii</i> (Matsumara)	105
	Article 5 : Cold acclimation triggers major transcriptional changes in <i>Drosophila suzukii</i> ..	135

Discussion générale	167
I. Rappel du contexte de l'étude	168
II. Thermotolérance de <i>Drosophila suzukii</i>	169
II.1. Thermotolérance basale.....	169
II.1.a. Tolérance au froid	169
II.1.b. Tolérance au chaud.....	170
II.1.c. La thermotolérance basale de <i>D. suzukii</i> permet-elle d'expliquer le succès de son invasion ?.....	171
II.2. Plasticité de la tolérance au froid	173
II.2.a. Effets des températures fluctuantes.....	173
II.2.b. Effets de l'acclimatation.....	175
II.2.c. Aparté sur les « morphes d'hiver »	176
III. Mécanismes sous-jacents à la plasticité de la tolérance au froid chez <i>D. suzukii</i>	177
III.1. Maintien de la structure des protéines.....	177
III.2. Adaptation homéovisqueuse des membranes.....	180
III.3. Activité ionique des membranes et maintien de la fonction neuromusculaire.....	181
III.4. Réserves lipidiques.....	183
III.5. Dormance reproductive	184
III.6. Modifications de l'activité des voies métaboliques	185
III.7. Maintien de l'homéostasie	186
IV. L'utilisation du froid en lutte biologique : Projet Suzukill	188
V. Conclusions et perspectives	191
Références	195
Annexes	235
Annexe 1	235
Annexe 2	251
Annexe 3	260
Annexe 4	279
Annexe 5	288

Liste des publications

- **Article 1 : Enriquez T.**, Colinet H. (2017). Basal tolerance to heat and cold exposure of the spotted wing drosophila, *Drosophila suzukii*. PeerJ 5: e3112.
- **Article 2 : Enriquez T.**, Ruel D., Charrier M., Colinet H. (2018). Effects of fluctuating thermal regimes on cold survival and life history traits of the spotted wing Drosophila (*Drosophila suzukii*). Insect science. doi:10.1111/1744-7917.12649
- **Article 3 : Enriquez T.**, Renault D., Charrier M., Colinet H. (2018). Cold acclimation favors metabolic stability in *Drosophila suzukii*. Frontiers in physiology, 9, 1506.
- **Article 4 : Enriquez T.**, Colinet H. (2019). Cold acclimation triggers lipidomic and metabolic adjustments in the spotted wing drosophila *Drosophila suzukii* (Matsumara). American Journal of Physiology - Regulatory, Integrative and Comparative Physiology. doi:10.1152/ajpregu.00370.2018
- **Article 5 : Enriquez T.**, Colinet H. (2019). Cold acclimation triggers major transcriptional changes in *Drosophila suzukii*. BMC Genomics, 20(1),413.
- **Annexe 1 : Nikolouli K.**, Colinet H., Renault D., **Enriquez T.**, Mouton L., Gibert P., ... Bourtzis K. (2017). Sterile insect technique and Wolbachia symbiosis as potential tools for the control of the invasive species *Drosophila suzukii*. Journal of pest science, 91(2), 489-503.

Liste des abréviations

AA :	acclimatation au stade adulte
DA :	acclimatation développementale
DA+AA :	combinaison acclimatation développementale et au stade adulte
DRO :	dérivés réactifs de l'oxygène
GO-term :	gene ontology term
GPChol :	phosphocholines
GPEtn :	phosphoéthanolamines
GPL :	glycérophospholipides
Lyso-GPL :	lyso-glycérophospholipides
PCT :	protéines de choc thermique
PE/PC :	ratio GPEtn/GPChol
RTF :	régimes de températures fluctuantes
TAG :	triacylglycerides
TFC :	températures froides constantes
TIS :	technique de l'insecte stérile

Liste des figures

Figure 1 : Schéma représentant les grandes étapes des invasions biologiques.	4
Figure 2 : Variations théoriques de la température corporelle d'organismes homéothermes et poïkilothermes.	6
Figure 3 : Courbes de performance thermique.	7
Figure 4 : Présentation des différentes stratégies de tolérance au froid des insectes.	9
Figure 5 : Normes de réaction et plasticité phénotypique.	12
Figure 6 : Représentation des trois phases de fluidité de la bicouche phospholipidique.	21
Figure 7 : Causes physiologiques des dégâts provoqués par les températures froides, et mécanismes contrant ces effets.	25
Figure 8 : Représentation schématique des différentes structures des phospholipides dans les membranes cellulaires.	27
Figure 9 : Présentation des critères d'identification de <i>Drosophila suzukii</i>	33
Figure 10 : Arbre phylogénétique du sous-genre <i>Drosophila</i>	33
Figure 11 : Aire de répartition actuelle de <i>Drosophila suzukii</i>	35
Figure 12 : Cycle de vie de <i>Drosophila suzukii</i>	36
Figure 13 : Organisation des chapitres, questions et hypothèses.	42
Figure 14 : A : Courbe de performance de <i>D. suzukii</i> ; B : Comparaison des $C_{t_{min}}$ (°C) de <i>D. suzukii</i> et d'autres espèces du groupe melanogaster (et de <i>Drosophila montana</i> , du groupe virilis)	170
Figure 15 : Maturation des ovocytes de femelles <i>D. suzukii</i> témoins ou acclimatées.	184
Figure 16 : Schéma récapitulatif des résultats concernant les mécanismes sous-jacents de la plasticité phénotypique chez <i>D. suzukii</i>	188
Figure 17 : Impact des conditions de transport après irradiation sur la qualité de <i>D. suzukii</i>	190

Liste des tableaux

Tableau 1 : Exemples de taxons montrant une augmentation de la tolérance au froid liée à l'acclimatation développementale, graduelle ou rapide.	17
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Introduction générale

Avant-propos

Drosophila suzukii est une espèce de drosophile, originaire de l'Est de l'Asie, récemment invasive en Europe, Amérique du Nord et Amérique du Sud (Asplen et al., 2015; Cini et al., 2012; Gibert et al., 2016; Hauser, 2011). Les larves de cette mouche se développent sur les fruits mûrs, occasionnant des dégâts importants sur une large gamme de productions fruitières. Les baisses de rendement cumulées au coût de sa gestion font que *D. suzukii* a un impact économique extrêmement important (Bolda et al., 2010; De Ros et al., 2015; Goodhue et al., 2011). *Drosophila suzukii* parasite également des hôtes sauvages, et pourrait rentrer en compétition avec des espèces natives consommant ces fruits. Il est donc possible qu'elle ait un impact non négligeable sur la biodiversité locale dans les aires nouvellement colonisées. Pour lutter contre ce ravageur, les producteurs ont généralement recours à une utilisation intensive de pesticides (Haviland and Beers, 2012), ajoutant une pression supplémentaire sur l'environnement. Afin de lutter efficacement contre ce ravageur, il est important d'avoir de solides connaissances de sa biologie. Le succès invasif de cette espèce étant en partie dû à sa capacité à moduler sa thermotolérance, il est nécessaire d'étudier cet aspect, et notamment les mécanismes physiologiques sous-jacents à cette plasticité.

Cette introduction sera divisée en trois parties. Dans une première partie je présenterai les problématiques posées par les espèces invasives, particulièrement les insectes, en mettant en évidence l'importance de la plasticité de leur tolérance au froid dans le succès de leur invasion. Je décrirai ensuite les bases physiologiques de la plasticité de la thermotolérance chez les insectes. Enfin, je présenterai le modèle d'étude de ma thèse, *D. suzukii*. J'aborderai les facteurs liés au succès de son invasion, avant de faire le point sur les connaissances concernant sa biologie thermique.

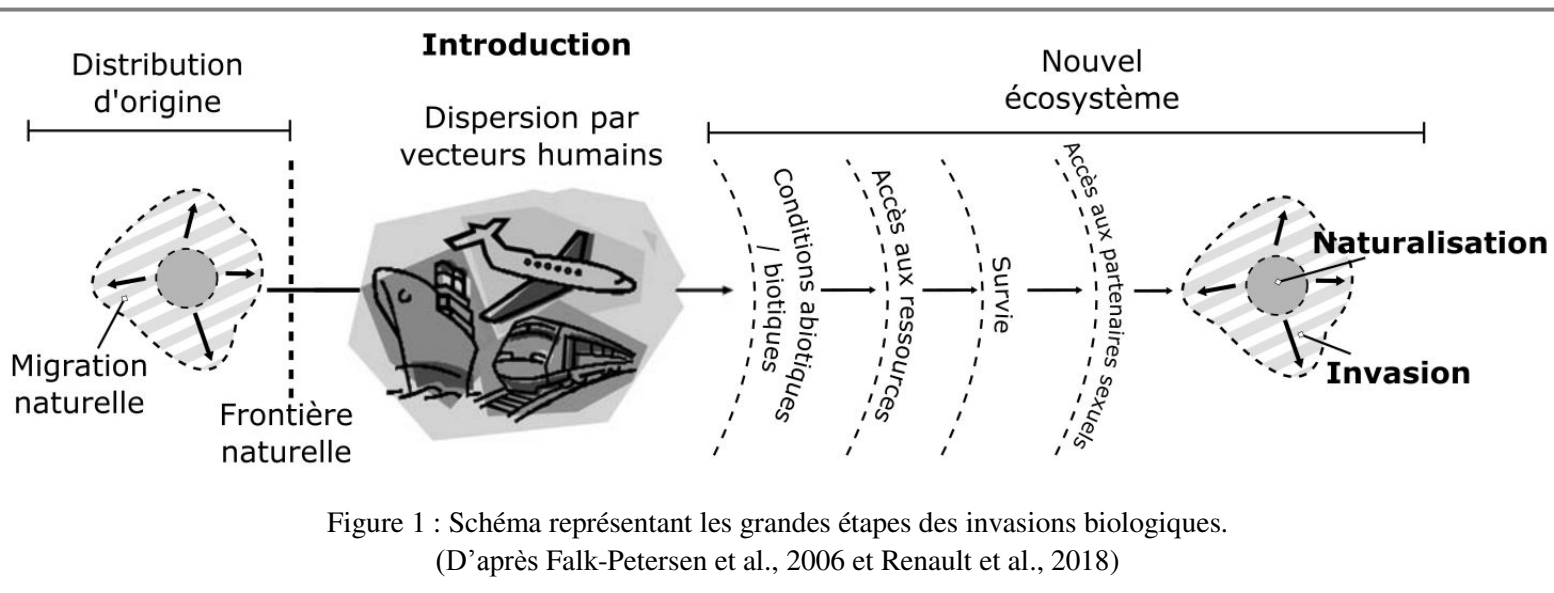
I. La plasticité de la thermotolérance, un facteur déterminant du succès des invasions biologiques

I.1. La problématique des espèces invasives

Notre écosystème subi actuellement une perte massive de biodiversité, qualifiée de sixième grande extinction, qui serait liée aux activités humaines (Barnosky et al., 2011). Parmi les nombreuses causes de cette érosion, les invasions biologiques jouent un rôle important (Lowe et al., 2000; Pimentel et al., 2005; Vitousek et al., 1997). Une espèce est qualifiée d'invasive lorsque suite à son introduction par un vecteur lié aux activités humaines, elle est implantée dans un nouvel environnement, s'y reproduit et qu'elle est capable d'étendre son aire de répartition (Falk-Petersen et al., 2006 , Figure 1). Ces espèces doivent donc tolérer les conditions abiotiques (température, hygrométrie, salinité, pH...) mais aussi biotiques (ressources nutritionnelles, compétition avec les espèces natives, prédation...) de ce nouvel environnement (Davis, 2009).

Les espèces invasives peuvent impacter négativement les espèces natives par modification de l'habitat colonisé (cas de la plante *Miconia calvescens* à Tahiti ; Meyer and Florence, 1996), par compétition (comme la coccinelle asiatique, *Harmonia axyridis* entrant en compétition avec les autres espèces aphidiphages en Europe et aux Etats-Unis ; Koch, 2003) ou encore par prédation (impact du serpent « brown tree snake » *Boiga irregularis* sur plusieurs espèces endémiques de l'île de Guam ; Rodda and Fritts, 1992). Les espèces invasives peuvent également représenter des menaces sanitaires, comme par exemple le moustique tigre *Aedes albopictus* qui est un important vecteur de maladies (Gratz, 2004).

Les espèces invasives ont également un impact important sur les systèmes agricoles. En effet, plusieurs milliers d'espèces, pathogènes de plantes, plantes adventices, organismes vertébrés et arthropodes ont envahi les différents agroécosystèmes et engendrent des pertes économiques considérables (Jardine and Sanchirico, 2018; Oerke, 2006; Pimentel et al., 2005). De plus, l'utilisation massive de pesticides pour lutter contre les espèces invasives représente une pression supplémentaire pour la biodiversité locale (Pimentel et al., 2005).



I.2. La température : un facteur fondamental influençant la biologie des organismes

Un des objectifs fondamentaux de l'écologie est de comprendre l'abondance et la distribution des espèces. Le concept de niche écologique permet d'appréhender ce problème. Hutchinson (1957, 1978) définit la niche écologique d'un organisme comme un espace multivarié dont chaque axe correspond à un paramètre de l'environnement de cet organisme, influant sur sa valeur adaptative (la valeur adaptative d'un individu est définie comme la capacité à produire des descendants matures par rapport à la moyenne de la population ; Ridley, 2004). Cette définition permet de distinguer la niche écologique fondamentale, qui correspond aux conditions abiotiques et aux ressources qui permettent à l'organisme de survivre et de se reproduire en l'absence d'interactions biotiques, de la niche réalisée qui inclut les interactions biotiques. La niche fondamentale permet donc de définir la distribution théorique de l'espèce considérée, tandis que la niche réalisée, plus restreinte, définit l'aire effectivement occupée par cette espèce. L'étude de la tolérance des organismes aux conditions abiotiques de l'environnement est par conséquent primordiale pour établir leur niche fondamentale et comprendre leur distribution, ce qui s'avère particulièrement important dans le cadre des invasions biologiques.

“Unlike many other variables that concern biologists, temperature is not just a property of life; it is a property of matter. Nothing escapes its control”. Angilletta, dans l'introduction de son livre « Thermal Adaptation – A Theoretical and Empirical Synthesis » (2009), souligne par

cette assertion que la température est un facteur abiotique influant largement sur la biologie des organismes. En effet, étant donné que la température définit la cinétique des réactions biochimiques et enzymatiques (Hochachka and Somero, 2002), elle agit à tous les niveaux fonctionnels, de la physiologie des organismes à l'échelle de la population. Par conséquent, la tolérance thermique (basale et induite, ces points seront développés par la suite dans la **partie I.7**) des organismes façonne leur niche fondamentale et influe donc sur leur répartition et leur capacité d'invasion (Bates et al., 2013; Kolar and Lodge, 2002; Zerebecki and Sorte, 2011).

I.3. Les différentes stratégies de thermorégulation

Les organismes sont divisés en deux catégories suivant leur type de réponse aux variations environnementales : les conformeurs qui subissent les changements imposés par le milieu extérieur, et les régulateurs qui possèdent des mécanismes physiologiques qui leur permettent de maintenir l'état de leur environnement interne malgré les changements imposés par le milieu (Willmer et al., 2004). Si la variable de l'environnement considérée est la température, on distinguera donc les organismes dits poïkilothermes, dont la température interne est directement dictée par les variations de la température de l'environnement, des organismes dits homéothermes, qui sont capables de réguler leur température interne, qui reste constante et ne change pas avec les variations de température du milieu (Angilletta, 2009 ; Figure 2). De manière générale, les poïkilothermes sont donc des organismes ectothermes : leur température corporelle dépend de sources de chaleur provenant de l'environnement, alors que les homéothermes sont pour la plupart des organismes endothermes : leur chaleur corporelle est produite et régulée par leur propre métabolisme (Bligh and Johnson, 1973).

I.4. Les courbes de performance

Un grand nombre de traits de vie sont affectés par les variations thermiques. La valeur de ces traits en fonction de la température du milieu est décrite par une courbe de performance (une norme de réaction ayant comme variables la température de l'environnement et la performance d'un trait, Figure 3A). Angilletta (2009) définit la performance comme la mesure d'un trait permettant d'estimer la capacité d'un organisme à fonctionner. Cette courbe de performance correspond à la gamme de température qu'un organisme peut tolérer (Huey and Stevenson, 1979) : la performance de l'organisme est maximale lorsque la température est proche de la température optimale (T_{opt}), puis décroît à mesure que la température diminue ou augmente, jusqu'à atteindre des zones de performances sub-optimales. Lorsque la température continue de varier, la performance continue de décroître jusqu'à atteindre des zones de stress

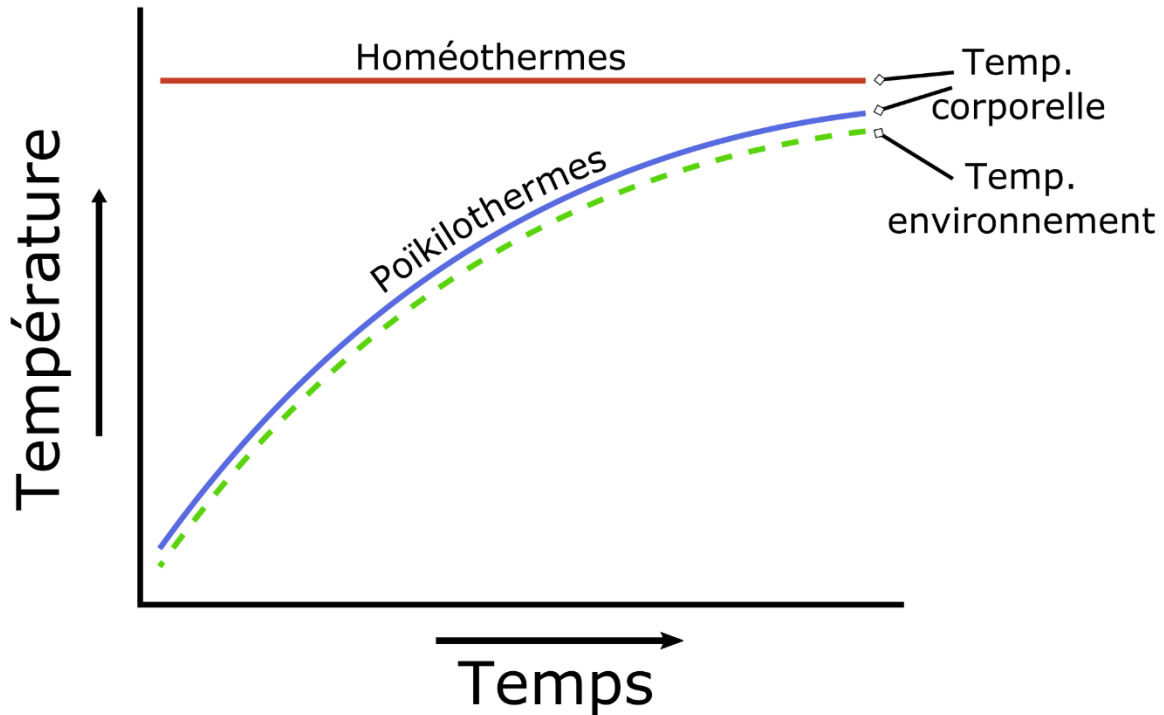
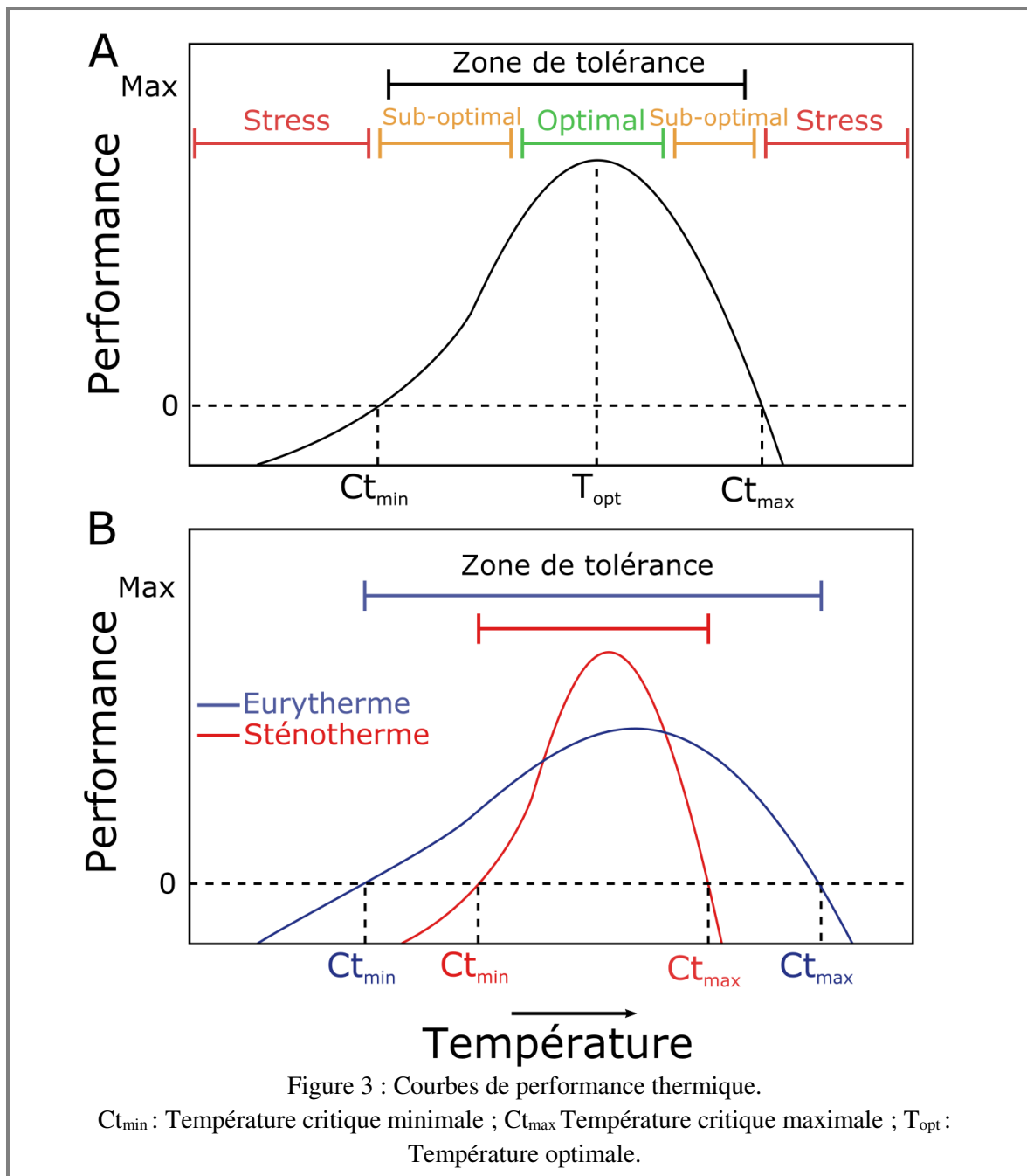


Figure 2 : Variations théoriques de la température corporelle d'organismes homéothermes et poïkilothermes. La courbe verte pointillée représente la variation de la température de l'environnement en fonction du temps. La courbe bleue représente l'évolution temporelle de la température corporelle d'organismes poïkilothermes, qui suit la température de l'environnement. La courbe rouge représente la température corporelle d'organismes homéothermes, dont la température est régulée et varie indépendamment de la température environnementale.

thermique, délimitées par les températures critiques minimale et maximale ($C_{t_{min}}$ et $C_{t_{max}}$; Hazell and Bale, 2011), qui correspondent à la température à laquelle une perte totale de fonction du trait est observée (généralement corrélée à une perte de la fonction neuromotrice). Pour bien comprendre à quoi correspond un stress thermique, nous pouvons nous référer à la définition établie par Korsloot et al. (2004), qui définissent le stress comme un événement subi par un organisme, provenant d'un ou plusieurs facteurs environnementaux (dans le cas présent : la température) altérant sa valeur adaptative.

Les courbes de performances sont généralement asymétriques : la performance d'un organisme diminue progressivement lorsque la température baisse, mais lorsqu'elle augmente, la baisse de performance est plus abrupte (Martin and Huey, 2008). Certains organismes, dit eurythermes, sont généralistes, et sont capables de tolérer une large gamme de température, au contraire des sténothermes qui ne peuvent tolérer que des gammes de température restreintes (Somero et al., 1996 , Figure 3B).



I.5. Les stratégies de tolérance au froid des insectes

Les insectes sont présents dans la plupart des écosystèmes terrestres et sont donc soumis à des conditions environnementales très variées. La grande majorité des insectes sont poïkilothermes, à l'exception de quelques espèces provenant de différents taxons (Lépidoptères, Odonates, Hyménoptères, Diptères, Coléoptères...) qui sont capables de produire leur propre chaleur interne en faisant activement fonctionner leurs muscles thoraciques ou ailes (Heinrich, 1993; May, 1979). En raison de la petite taille et de la faible masse des

insectes, leur température corporelle varie très fortement avec les variations thermiques de leur milieu. La température faisant partie des composantes fondamentales de la niche écologique d'une espèce (voir **partie I.2**), la thermotolérance des insectes influence grandement leurs aires de répartition et leurs traits de vie (Addo-Bediako Abraham et al., 2000; Bale, 2002; Kimura, 2004; Sunday et al., 2011). La relation entre la thermotolérance et la biogéographie est particulièrement évidente lorsque l'on observe la corrélation entre les températures minimales qu'une espèce peut tolérer et les températures minimales de son environnement (Addo-Bediako Abraham et al., 2000; Kellermann et al., 2012a; Kimura, 2004). En conséquence, de nombreux travaux se sont intéressés à la thermotolérance des insectes et notamment aux bases physiologiques déterminant les fortes variations de tolérance au froid que l'on retrouve au sein de ces taxons.

La thermotolérance des insectes a été largement étudiée et différentes stratégies de tolérance au froid ont été définies (Bale, 1987; Denlinger and Lee Jr, 2010; Overgaard and MacMillan, 2017; Sinclair, 1999; Vannier, 1994). Les premiers travaux décrivant la tolérance au froid des insectes classifiaient ces derniers selon leur capacité à tolérer la formation de gel extracellulaire : tolérant au gel ou intolérant au gel (freezing tolerant / intolerant ; Salt, 1936, 1961). Toutefois cette classification est loin d'être suffisante. En effet un grand nombre d'insectes succombent à des températures bien supérieures aux températures provoquant la formation de gel, comme par exemple la mouche tsé-tsé *Glossina pallidipes* qui montre en conditions expérimentales une $C_{t_{min}}$ de 21°C (Terblanche et al., 2007a). Il est donc nécessaire de différencier les insectes possédant des stratégies leur permettant de tolérer des températures extrêmes de ceux succombant à des expositions à des températures froides modérées. La communauté scientifique s'accorde actuellement sur la définition de quatre stratégies de thermotolérance chez les insectes, qui sont présentées en Figure 4 (Lee Jr, 1989, 2010; Overgaard and MacMillan, 2017; Vannier, 1994).

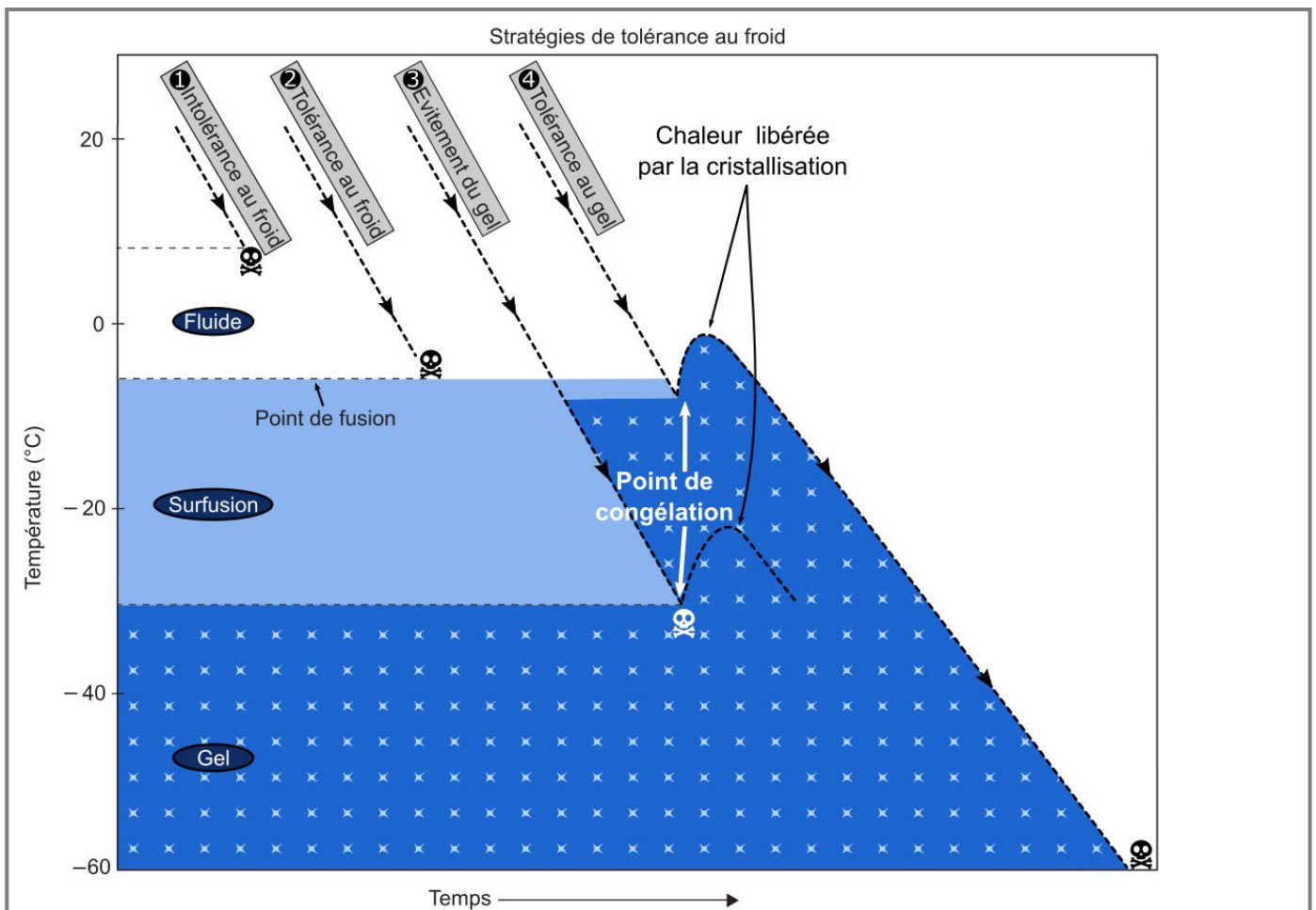


Figure 4 : Présentation des différentes stratégies de tolérance au froid des insectes.

Les zones de couleurs différentes correspondent aux états physiques de l'hémolymphe en fonction de la température corporelle des insectes (représentée par les lignes noires pointillées), qui est abaissée au cours du temps.

- ① Ces insectes succombent lors d'une exposition courte à des températures modérément froides (proches de 0°C) : **Intolérance au froid** (« chill susceptible »).
- ② Ces insectes sont capables de survivre à des expositions prolongées à des températures inférieures à 0°C, mais supérieures au point de congélation : **Tolérance au froid** (« chill tolerant »).
- ③ Ces insectes survivent tant qu'il n'y a pas formation de gel dans leur organisme, la mort survient au moment de la congélation. Lorsque leur température corporelle atteint le point de fusion de l'hémolymphe, cette dernière rentre dans un état de surfusion : elle reste à l'état liquide bien que sa température soit en dessous de sa température de congélation. Leur survie est définie par leur capacité de surfusion qui empêche la formation de cristaux de glace (cette capacité dépend de la taille du corps de l'insecte, de l'eau qui y est contenue, et de l'accumulation de solutés à faibles poids moléculaires ; Lee Jr, 2010) : **Evitement du gel** (« freeze avoiding »). La détermination de la température de congélation (« super cooling point » ; caractérisée par un pic de température dû à l'exotherme de la cristallisation) est donc particulièrement pertinente pour décrire la stratégie de survie de ces espèces.
- ④ Ces insectes synthétisent des agents de nucléation (protéines ou lipoprotéines) qui catalysent la formation de cristaux de glace dans le milieu extracellulaire lorsque leur température corporelle atteint des températures d'approximativement -5, -10°C. Cette formation de glace extracellulaire permet d'éviter la formation de gel intracellulaire et protège ainsi la cellule jusqu'à des températures extrêmement froides (jusqu'à -70°C ; Lee Jr, 1989) : **Tolérance au gel** (« freeze tolerant »).

D'après Vannier, 1994, Lee Jr, 1989, Overgaard et MacMillan, 2017.

Etant donné que la plus grande diversité d'insectes se trouve dans les régions tropicales, il est possible de considérer que la plupart des espèces sont intolérantes au froid et que les stratégies de survie ou d'évitement du gel sont les moins répandues (Overgaard and MacMillan, 2017). L'analyse de ces différentes stratégies démontre que les insectes sont capables de tolérer des gammes de températures extrêmement larges, ce qui leur permet de tolérer des conditions environnementales très variées, expliquant leur présence dans la quasi-totalité des écosystèmes terrestres.

I.6. Adaptation de la tolérance au froid chez les insectes

Lorsque les insectes sont soumis à des variations thermiques, des adaptations progressives de leur thermotolérance peuvent se mettre en place via des processus évolutifs. Ces adaptations nécessitent un nombre important de générations pour se mettre en place, et requièrent une importante diversité génétique dans la population (Bradshaw and Holzapfel, 2010; Hoffmann et al., 2003b; Huey, 2010; Overgaard et al., 2010). L'augmentation de la tolérance au froid de différentes populations d'une même espèce avec la latitude ou l'altitude illustre la sélection des phénotypes par les contraintes climatiques (Addo-Bediako Abraham et al., 2000; Gaston and Chown, 1999; Gibert and Huey, 2001; Hoffmann et al., 2003b; Huey, 2010; Zani et al., 2005). Ces différences de thermotolérance persistent lorsque des individus d'une même espèce prélevés le long d'un gradient latitudinal sont élevés en condition de laboratoire à des températures similaires, ce qui confirme que ces différences de thermotolérance possèdent des bases génétiques (Ayrinhac et al., 2004; Hallas et al., 2002). De plus, des expériences de sélection en laboratoire ont pu démontrer l'héritabilité de ce trait (Anderson et al., 2005; Bertoli et al., 2010).

I.7. La plasticité de la tolérance au froid des insectes

I.7.a. Définition de la plasticité phénotypique

La mise en place des réponses évolutives nécessite un nombre important de générations. Toutefois, la thermotolérance individuelle des insectes n'est pas figée tout du long de leur vie. En effet, plusieurs facteurs abiotiques, comme l'humidité relative (Bubliy et al., 2012; Kobey and Montooth, 2013; Terblanche et al., 2011), ou biotiques comme l'âge (Bowler and Terblanche, 2008) ou le stade de développement, influencent leur thermotolérance (Jensen et al., 2007; Krebs and Loeschcke, 1995). La thermotolérance peut également être ajustée plus rapidement, afin de faire face à des variations de température quotidiennes ou saisonnières, grâce à la plasticité phénotypique. Les prochains paragraphes seront par conséquent consacrés

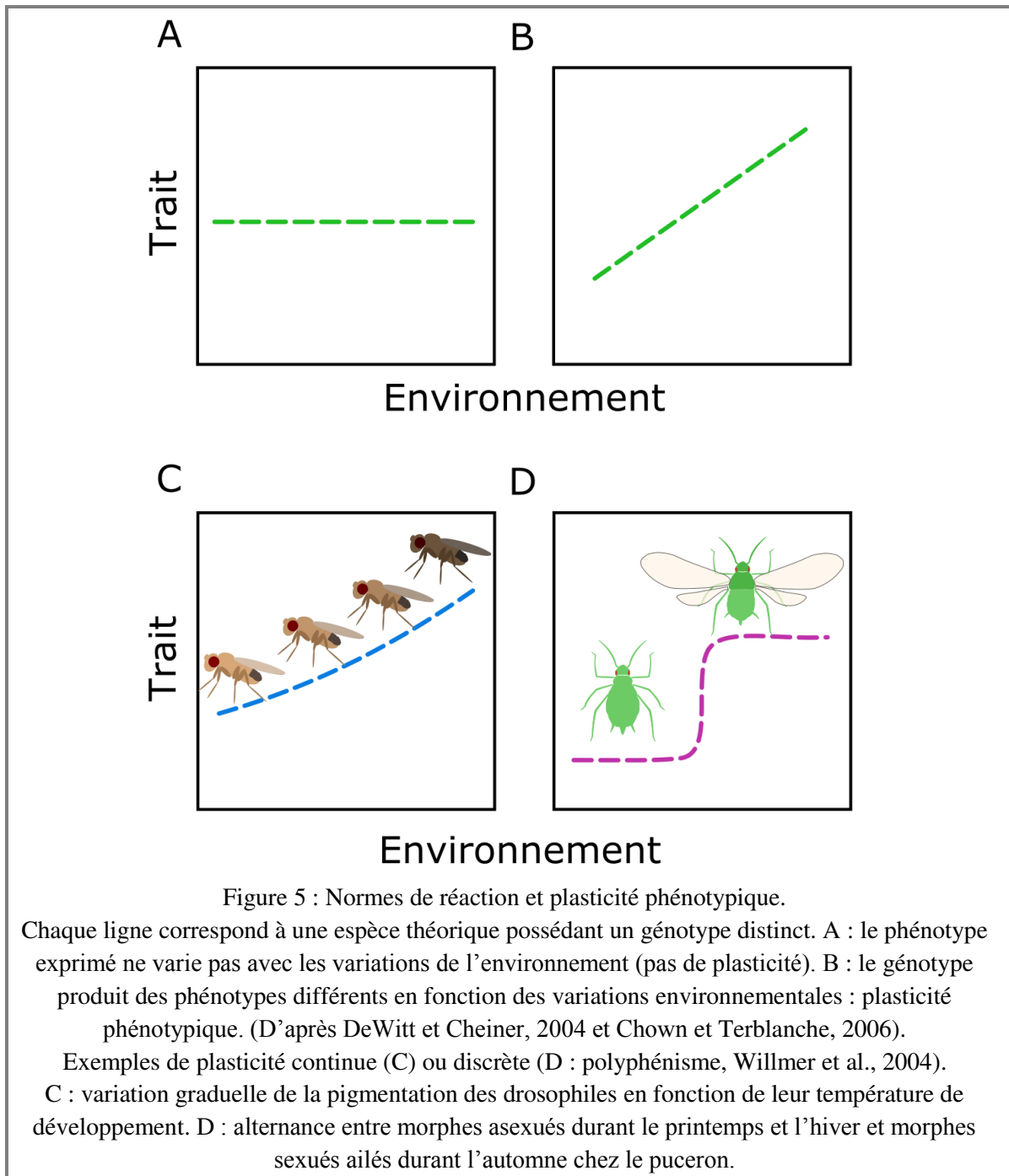
à la plasticité de la thermotolérance chez les insectes. Il est toutefois important de préciser que même si ici l'adaptation et la plasticité phénotypique sont présentées dans des paragraphes séparés, la plasticité phénotypique est indissociable de la sélection naturelle. En effet, plusieurs études considèrent que la plasticité phénotypique est un trait parmi d'autres, permettant des réponses adaptatives (Gabriel, 2005; Trussell and Smith, 2000) et qu'elle est par conséquent également soumise à la sélection naturelle (Dietz and Edwards, 2006; Richards et al., 2006; Schlichting, 1986; West-Eberhard, 1989).

La plasticité phénotypique correspond à la capacité d'un génotype à exprimer différents phénotypes en réponse à des variations environnementales (Chown and Terblanche, 2006; DeWitt and Scheiner, 2004; Stearns, 1989, Figure 5). L'ensemble de ces phénotypes exprimés forme une norme de réaction (Via et al., 1995).

Selon certains auteurs, la plasticité phénotypique *stricto sensu* correspond à la plasticité développementale, qui regroupe les ajustements phénotypiques irréversibles produits par l'environnement durant l'ontogénèse (Chown and Nicolson, 2004; Piersma and Drent, 2003). La loi taille-température est un bon exemple de plasticité développementale : chez des ectothermes en conditions de laboratoire, la taille des individus augmente avec la diminution de la température subie durant le développement (Atkinson, 1996). Cette observation dérive de la règle de Bergmann qui met en évidence au sein d'une même espèce l'augmentation de la taille des individus dans des zones de climat froid (Bergmann, 1848; Johnston and Bennett, 1996). Les modifications induites par la plasticité phénotypique peuvent se mettre en place de manière continue, décrivant une norme de réaction linéaire ou polynomiale (David et al., 1990), ou de manière discrète, il s'agit alors de polyphénisme (Figure 5C et D ; Braendle et al., 2006).

Les ajustements provoqués par la plasticité peuvent être également réversibles, on parle alors de flexibilité phénotypique (Piersma and Drent, 2003; Piersma and Gils, 2011). C'est le cas de certaines bactéries qui sont capables de produire des enzymes différentes en fonction du substrat qu'elles rencontrent (Tollrian and Harvell, 1999), ou encore du lagopède alpin dont la couleur du plumage varie en fonction de la saison (Montgomerie et al., 2001).

La plasticité influence une grande variété de traits des organismes, aussi bien physiologiques, morphologiques que comportementaux (DeWitt and Scheiner, 2004). La tolérance au froid des insectes est également largement modulée par la plasticité. Ces réponses seront développées dans les sections suivantes.



I.7.b. Ajustements comportementaux

Lorsqu'un insecte est confronté à des variations stressantes de température, la mise en place de réponses comportementales, comme l'évitement (spatial, ou temporel) du stress, lui permet de maintenir sa température corporelle dans son optimum thermique (voir Figure 3 ; Abram et al., 2017). Ces stratégies, rapides à mettre en place et très efficaces, sont

déterminantes pour la survie des insectes face à un stress thermique (May, 1979; Stevenson, 1985).

Il existe un grand nombre de stratégies de thermorégulations comportementales (Abram et al., 2017; Stevenson, 1985). Certains insectes présentent des comportements migratoires (à courte ou large échelle) permettant d'éviter les conditions délétères de l'environnement (Johnson, 1969; Kennedy, 1961). La migration du papillon monarque *Danaus plexippus* est un des exemples les plus marquants de migration sur de larges distances chez les insectes. Le monarque ne tolère pas les températures hivernales d'Amérique du Nord. La réduction de la photopériode en automne provoque la migration des adultes, qui entreprennent un voyage sur près de 3000 km, jusqu'aux sites d'hivernage au Mexique et au sud de la Californie, où ils rentreront en diapause. En février, les accouplements ont lieu et ce sont les générations suivantes qui reprendront la migration pour retourner en Amérique du Nord (Dingle, 2009; Tuskes and Brower, 1978; Urquhart and Urquhart, 1978).

A une échelle plus fine, les insectes sont capables d'éviter un stress thermique en choisissant un microclimat tamponné (Coulson et al., 1995; Danks, 1991; Hawes et al., 2008; Pincebourde et al., 2016; Woods et al., 2015). Par exemple, les larves des drosophiles cactophiles du désert de Sonora s'enfouissent dans le sol proche des cactus, où les conditions de températures et d'hygrométrie sont plus favorables en comparaison à l'air chaud et sec du désert (Gibbs et al., 2003). On peut également mentionner l'exemple des lépidoptères pondant leurs œufs sur leur plante hôte où ils sont protégés des conditions environnementales par la couche limite des feuilles (Potter et al., 2009).

Ces stratégies de plasticité comportementale, reposant sur l'hétérogénéité du milieu sont essentielles à la survie des insectes et montrent donc une véritable pertinence écologique. Toutefois ce trait est peu pris en compte dans les études biogéographiques (Sunday et al., 2014).

I.7.c.L'acclimatation au froid

I.7.c.i. Définition

En parallèle à la plasticité comportementale, les insectes ont la capacité de mettre en place d'autres types de réponses, permettant l'augmentation de leur thermotolérance, comme les processus d'acclimatation. L'acclimatation correspond aux ajustements physiologiques, biochimiques, ou développementaux en réponse à un stimulus environnemental. Pour désigner l'acclimatation thermique, certains auteurs se réfèrent aux conséquences phénotypiques engendrées (*i.e.* l'acquisition de la thermotolérance), et d'autres aux causes des changements

phénotypiques (*i.e.* le traitement). Dans cette thèse, j'utiliserai la deuxième définition. Ainsi, l'acclimatation thermique correspond à une exposition à des températures suboptimales avant un stress thermique provoquant une augmentation de la thermotolérance (voir Figure 3A pour les notions de stress et de températures suboptimales). L'acclimatation est donc une forme de plasticité phénotypique (Angilletta, 2009). Cette réponse est également référencée dans la littérature sous le terme de thermotolérance induite (Chown and Nicolson, 2004).

I.7.c.ii. L'acclimatation chez les insectes

De nombreuses études se sont intéressées à l'acclimatation chez les insectes, qui existe sous de nombreuses formes et a fait l'objet de nombreuses études (Angilletta, 2009; Chown and Nicolson, 2004). Nous nous concentrerons sur les trois formes d'acclimatation suivantes, qui sont les plus répandues dans la littérature : l'acclimatation développementale, l'acclimatation graduelle, et l'acclimatation rapide (« rapid cold hardening »), qui se différencient par le stade de développement durant lequel l'acclimatation est appliquée, la durée du traitement et l'intensité de la température subie.

L'acclimatation développementale correspond à un processus d'acclimatation subi durant l'ontogénèse (Colinet and Hoffmann, 2012; Teets and Denlinger, 2013). Pour certains auteurs, les effets transgénérationnels font partie de l'acclimatation développementale, incluant donc les réponses des parents à l'acclimatation qui sont transmises à leur descendance, augmentant leur tolérance thermique (Angilletta, 2009). Toutefois seuls les effets intragénérationnels de l'acclimatation développementale seront développés dans ce manuscrit. Les variations de température durant l'ontogénèse induisent des modifications profondes d'un grand nombre de traits, qui sont considérées comme irréversibles (Chown and Nicolson, 2004; Piersma and Drent, 2003 ; voir **partie I.7.b**). L'augmentation de la taille des individus, ou l'augmentation de la mélanisation en réponse aux baisses de température durant le développement illustrent le caractère irréversible de la plasticité développementale (Piersma and Drent, 2003 , voir Figure 5C). Toutefois d'autres traits sont réversibles (Piersma and Drent, 2003). Ainsi, pour la tolérance thermique, des phénomènes de désacclimatation peuvent se mettre en place si la température change après l'acclimatation développementale, la thermotolérance s'ajustant aux conditions subies au stade adulte (Fischer et al., 2010; Slotsbo et al., 2016; Zeilstra and Fischer, 2005).

L'acclimatation graduelle désigne une période d'acclimatation de moyen terme (plusieurs jours), subie le plus souvent au stade adulte (Colinet and Hoffmann, 2012; Teets and Denlinger, 2013).

Contrairement à l'acclimatation développementale ou à l'acclimatation graduelle, qui sont respectivement induites par des expositions à long et moyen termes, l'acclimatation rapide est un processus provoqué par des périodes d'expositions brèves (expositions de quelques heures, généralement une ou deux heures), à des températures relativement stressantes pour l'organisme étudié (Lee et al., 1987). L'acclimatation rapide diffère des processus précédemment présentés par différents aspects. En effet, même si l'augmentation de la tolérance au froid est importante après acclimatation rapide, elle n'est pas suffisante pour supporter des températures extrêmes (ex : inférieures à -25°C), contrairement à l'acclimatation à plus long terme (Lee and Denlinger, 2010). De plus, l'augmentation de la tolérance au froid produite par acclimatation rapide retourne à un niveau basal peu de temps après la phase d'acclimatation (Li, 1999; McDonald et al., 1997).

Lorsque ces différents types d'acclimations sont combinés, l'effet sur la tolérance au froid est cumulatif (Colinet and Hoffmann, 2012; Kelty and Lee, 2001). Par exemple l'acclimatation rapide induit une augmentation de thermotolérance plus importante lorsque les insectes sont préalablement acclimatés (acclimatation développementale ou graduelle) au froid (Colinet and Hoffmann, 2012; Powell and Bale, 2005; Shintani and Ishikawa, 2007). Ces effets cumulatifs montrent que chaque type d'acclimatation possède des mécanismes qui leurs sont en partie propres (Colinet and Hoffmann, 2012; Gerken et al., 2015; Teets and Denlinger, 2013).

1.7.c.iii. Le rôle de l'acclimatation

L'augmentation de la tolérance au froid provoquée par l'acclimatation développementale, graduelle, ou rapide a été largement étudiée chez l'espèce modèle *Drosophila melanogaster*, ainsi que chez un grand nombre d'autres taxons d'insectes (voir Tableau 1). Le fait que ces réponses plastiques soient largement répandues chez les insectes démontre leur importance d'un point de vue écologique. Ces différents processus d'acclimatation permettent la mise en place d'ajustements à plus ou moins long terme, après exposition à des températures modérées ou extrêmes, ce qui confère un avantage important aux insectes pour tolérer les variations de température de leur environnement. Des expériences ont d'ailleurs montré que les cycles quotidiens des températures environnementales induisaient des

réponses similaires à l'acclimatation rapide chez *D. melanogaster*, permettant des ajustements de la thermotolérance, soulignant la pertinence écologique de ce trait (Kelty and Lee, 2001; Overgaard and Sørensen, 2008).

A une échelle temporelle plus large, la thermotolérance des insectes est ajustée de manière saisonnière, comme lors de la diapause (Denlinger, 1991; Hodkova and Hodek, 2004; Tauber et al., 1986). La diapause est une réponse plastique saisonnière très répandue chez les insectes, définie comme une phase d'inactivité du comportement, de la morphogénèse, de la croissance et des fonctions de reproduction, et associée à une réduction de l'activité métabolique (Košťál, 2006). Durant la phase de diapause, des mécanismes augmentant la tolérance au froid des insectes se mettent en place (Denlinger, 1991; Tauber et al., 1986; Teets and Denlinger, 2013). La mouche galligène *Eurosta solidaginis* représente un bon exemple d'augmentation de la tolérance au froid en lien avec la diapause. Cet insecte d'Amérique du Nord, doit subir des températures extrêmes durant l'hiver. Les larves de stade 3 rentrent en diapause à l'intérieur de leur galle, leur permettant de survivre à ces conditions (Irwin et al., 2001). Cette période est corrélée avec une augmentation de la tolérance au froid, leur permettant de survivre à la congélation (Baust and Nishino, 1991; Bennett et al., 1997; Yi and Lee, 2005). Cette augmentation de la tolérance au froid pourrait être en lien avec l'acclimatation naturelle¹ (Denlinger, 1991; Hodkova and Hodek, 2004). En effet, lors de la diapause du gendarme *Pyrrhocoris apterus*, l'augmentation de la tolérance au froid se produit en deux étapes : lorsque la diapause est induite par la diminution de la photopériode, puis lors des baisses de température, suggérant une réponse liée à l'acclimatation naturelle (Hodkova and Hodek, 2004).

¹ Processus d'acclimatation en réponse à des stimulus environnementaux *in natura* (Smith and Dukes, 2013)

Tableau 1 : Exemples de taxons montrant une augmentation de la tolérance au froid liée à l'acclimatation développementale, graduelle ou rapide.

Ordre	Acclimatation	Stade	Exemple de références
Acari	Rapide	Adulte	(Broufas and Koveos, 2001)
Collembola	Rapide	Adulte	(Bahrndorff et al., 2009)
Orthoptera	Graduelle	Adulte	(Andersen et al., 2017)
	Rapide	Juvénile	(Wang and Kang, 2003)
	Développementale	Du juvénile à l'adulte	(Powell and Bale, 2006)
Hemiptera	Graduelle	Adulte	(Ju et al., 2011; Šlachta et al., 2013)
	Rapide	Juvénile; Adulte	(Larsen et al., 1993; Lee et al., 1987; Powell and Bale, 2004)
Thysanoptera	Rapide	Juvénile	(McDonald et al., 1997)
Lepidoptera	Développementale	De la larve à l'adulte; Chrysalide uniquement	(Chidawanyika and Terblanche, 2011; Zeilstra and Fischer, 2005)
	Graduelle	Adulte	(Larsen and Lee Jr, 1994; Zheng et al., 2011)
	Rapide	Larve ; Adulte	(Kim and Kim, 1997; Larsen and Lee Jr, 1994)
Coleoptera	Développementale	De l'œuf à l'adulte	(David et al., 1977; Scharf et al., 2015)
	Graduelle	Adulte	(David et al., 1977; Evans, 1983; Fields et al., 1998; Scharf et al., 2015; Smith, 1970)
	Rapide	Œuf ; Adulte	(Burks and Hagstrum, 1999; Lee et al., 1987; Shintani and Ishikawa, 2007; Sinclair and Chown, 2006; Terblanche et al., 2007b)
Diptera	Développementale	De l'œuf à l'adulte ; Larve uniquement	(Ayrinhac et al., 2004; Colinet and Hoffmann, 2012; Gibert and Huey, 2001; Košťál et al., 2011a; Meats, 1976; Parkash et al., 2014; Sisodia and Singh, 2010; Sørensen et al., 2016)
	Graduelle	Adulte	(Chen and Walker, 1994; Colinet et al., 2013, 2017a; Colinet and Hoffmann, 2012; Hoffmann and Watson, 1993; Kristensen et al., 2008; Rajamohan and Sinclair, 2009; Rako and Hoffmann, 2006)
	Rapide	Larve; Pupa; Adulte	(Coulson and Bale, 1990; Klok et al., 2003; Koveos, 2001; Lee et al., 1987; Lee Jr et al., 2006; Li, 1999; Nunamaker, 1993; Rosales et al., 1994; Terblanche et al., 2007b)

I.7.d. Les fluctuations thermiques

La plupart des travaux portant sur la thermotolérance des insectes utilisent des expositions à des températures constantes, ce qui permet de simplifier les schémas expérimentaux et de conclure quant à l'effet de la température testée. Toutefois, ces dernières années le nombre d'études portant sur les effets des variations de température pendant ces phases d'exposition a largement augmenté. Ces variations artificielles, induites en laboratoire, consistent en de brèves expositions à des températures optimales (un « pic » de chaleur) interrompant une période de stress thermique (Colinet et al., 2006; Javal et al., 2016; Košťál et al., 2007; Renault et al., 2004; Torson et al., 2015). Ce procédé est référencé dans la littérature sous l'appellation de régimes de température fluctuante (RTF). Les RTF conduisent à des réponses qui diffèrent singulièrement de celles observées en régimes constants (Colinet et al., 2015).

Les RTF permettent de diminuer, voir même de totalement compenser la mortalité normalement induite par la période de stress (Colinet et al., 2015, 2018). L'augmentation de la probabilité de survie est due à la mise en place de mécanismes de récupération pendant les phases de températures optimales, ainsi qu'à une augmentation de la thermotolérance liée à un processus d'acclimatation provoquée par les températures froides (Colinet et al., 2018; Grumiaux et al., 2019). Un grand nombre d'études ont montrés les effets bénéfiques des RTF sur la survie au froid des insectes, ainsi que sur d'autres traits d'histoires de vie (cf. Colinet et al., 2015, 2018). Ces effets positifs sur la survie, couplés au ralentissement du développement dû aux températures froides présentent également un fort intérêt appliqué. En effet, les RTF semblent particulièrement adaptés à la mise en place de protocole de stockage d'insectes à basses températures, pour la conservation de souches d'intérêt, le stockage d'auxiliaires (Colinet et al., 2006), ou de pollinisateurs (Rinehart et al., 2013).

Lors des pics de chaleur des RTF, le taux métabolique des insectes augmente nettement, ce qui suggère qu'une quantité importante d'énergie est consommée et est nécessaire à la mise en place des mécanismes physiologiques de récupération (Boardman et al., 2013; Lalouette et al., 2011; Yocum et al., 2011). Cette forte dépense d'énergie amènerait à des compromis énergétiques, qui pourraient avoir un effet négatif sur la valeur adaptative des individus (Takano, 2014), toutefois cette hypothèse n'a pas clairement été démontrée (Colinet et al., 2018).

Etant donné leur caractère artificiel, les RTF sont considérés comme non pertinents d'un point de vue écologique. Toutefois un grand nombre d'études montrent des réponses

extrêmement divergentes entre les régimes constants et fluctuants, sur un grand nombre de traits (survie, reproduction, longévité... ; Colinet et al., 2015). Ces divergences laissent sous-entendre que les réponses mises en place par les insectes dans leur environnement naturel, où les conditions thermiques varient quotidiennement, sont bien plus complexes que ce que les études basant leurs conclusions sur les températures constantes prédisent.

I.8. Le rôle de la plasticité de la tolérance au froid dans le succès des invasions biologiques

La pression propagulaire, qui inclut le nombre d'individus et le nombre d'événements d'introduction, est un facteur important dans le succès des invasions biologiques : plus cette pression est forte, plus la probabilité de succès de l'invasion est importante (Lockwood et al., 2005). Cependant, lors du processus d'invasion, les propagules sont soumises à une série d'étapes filtres : ces individus doivent survivre à la phase d'introduction, puis aux conditions du nouveau milieu, afin de pouvoir accéder à des ressources essentielles et s'y reproduire (Davis, 2009; Holway et al., 2002; Renault et al., 2018). Les propagules représentent une infime proportion de la population d'origine et sont donc par définition génétiquement appauvries, du fait d'un effet fondateur (Mayr, 1942; Provine, 2004). Cette faible diversité génétique peut limiter ces organismes dans leur capacité à tolérer les conditions abiotiques, comme la température, lors du transport ou dans leur nouvel environnement.

La plasticité phénotypique va compenser cette faible diversité en permettant aux organismes introduits d'ajuster leur physiologie, leurs comportements ou leur morphologie aux stress subis lors de la phase d'introduction et aux variations de température de leur nouveau milieu (Wilson et al., 2009). Dans la première partie de cette introduction, plusieurs exemples de plasticité de la tolérance au froid des insectes ont été présentés. Ces processus sont mis en place suite à des variations à différentes échelles de temps et à différentes intensités de températures, et provoquent des ajustements physiologiques qui permettent aux insectes de compenser les variations thermiques quotidiennes ou saisonnières de leur milieu. Ces mécanismes physiologiques seront développés dans la deuxième partie de cette introduction. La plasticité joue par conséquent un rôle déterminant dans le succès des invasions biologiques (Davidson et al., 2011; Nyamukondiwa et al., 2010; Renault et al., 2018). Le fait que la plupart des espèces invasives montrent une plasticité phénotypique supérieure aux espèces non invasives conforte ce point (Davidson et al., 2011).

Etudier la capacité des espèces invasives à tolérer les conditions de leur nouvel environnement et les ajustements physiologiques liés à la plasticité de la thermotolérance est donc essentiel à la compréhension du succès des invasions biologiques. Approfondir ces connaissances permettra à terme de mieux prédire l'évolution du front d'invasion et des variations démographiques des populations invasives d'une année sur l'autre afin d'adapter les moyens de luttés contre ces espèces.

II. Mécanismes physiologiques de la plasticité de la tolérance au froid

La plasticité de la thermotolérance chez les insectes possède des bases physiologiques complexes, qui sont présentées dans la **partie II.2**. Avant d'aborder ce point, il est toutefois nécessaire de comprendre la nature des dérèglements provoqués par les températures froides et leurs influences sur la physiologie des insectes.

II.1. Les dégâts provoqués par les températures froides

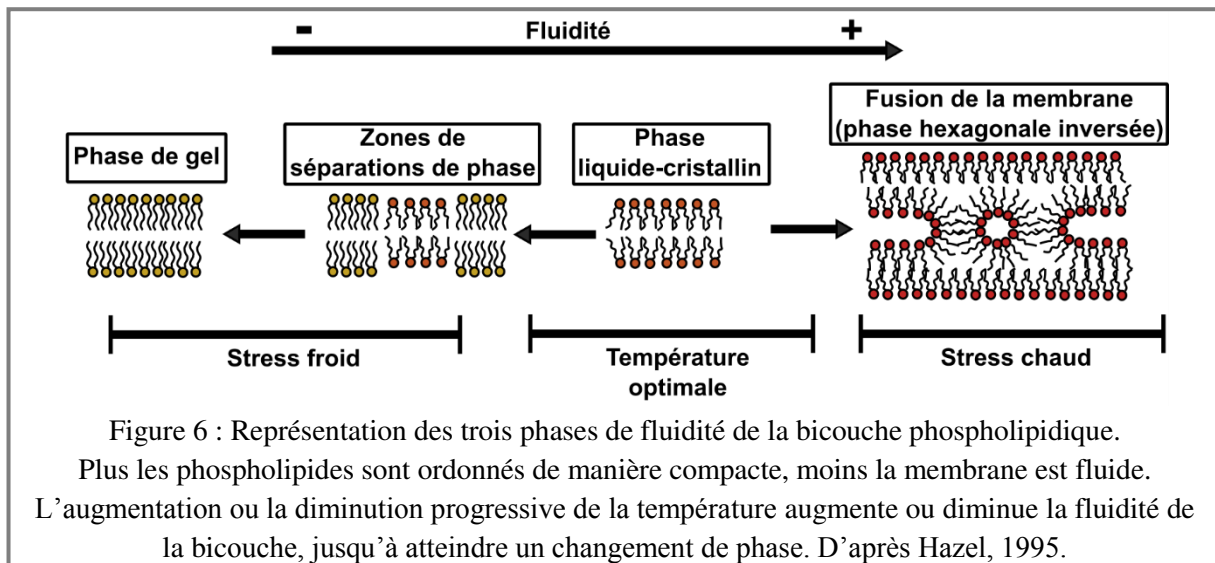
II.1.a. Stress froid extrême et formation de gel

Depuis les travaux de Salt (1936, 1961), de nombreuses études se sont intéressées à l'effet du gel sur la survie des insectes (Bale, 1987; Lee Jr, 1989, 2010; Pfister and Storey, 2006; Sinclair, 1999; Storey and Storey, 2005). La formation de cristaux de glace dans le milieu extracellulaire induit une réduction de la quantité de liquide, et donc une augmentation de la concentration des osmolytes, entraînant des mouvements de liquide hors des cellules dus à la forte pression osmotique, provoquant alors une importante déshydratation cellulaire (Lee Jr, 2010; Zachariassen, 1985). La formation des cristaux de glace à l'intérieur ou à l'extérieur des cellules peut également provoquer des dégâts physiques en perforant les membranes (Teets and Denlinger, 2013; Tursman and Duman, 1995) et compromettre l'intégrité des tissus en augmentant les espaces intercellulaires (Sinclair and Renault, 2010; Storey and Storey, 1988).

II.1.b. Dégâts directs et indirects provoqués par un stress froid modéré

Les expositions aux basses températures ne provoquant pas de formation de glace, ou froid modéré, (« chilling » en anglais) peuvent également induire des perturbations physiologiques complexes mettant à mal la survie des organismes (Hayward et al., 2014; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017).

Classiquement, les dégâts directs provoqués par des stress aigus (une brève exposition à des températures stressantes, mais ne provoquant pas la formation de gel) sont distingués des dommages indirects provoqués par des stress chroniques (exposition de plus longue durée à des températures modérées ; Chown and Nicolson, 2004; Lee Jr, 2010). Un stress aigu froid peut entraîner des effets directs en impactant la structure des macromolécules, telles que les membranes cellulaires, qui sont des structures thermosensibles (Cao-Hoang et al., 2010; Hazel, 1989, 1995). Les températures froides compromettent leur intégrité en diminuant la fluidité de



la bicouche phospholipidique, induisant dans les cas extrêmes des transitions de phases d'un état fluide (liquide-cristallin) à un état plus rigide (phase de gel lamellaire) et des zones de transitions de phases, provoquant une séparation latérale des lipides membranaires (Cossins, 1994; Hazel, 1995; Košťál, 2010; Lee Jr et al., 2006 ; voir Figure 6) . Un stress thermique peut également déstabiliser, voire dénaturer, la structure des protéines (Franks and Hatley, 1991; Todgham et al., 2007) et provoquer une désorganisation du cytosquelette (Cottam et al., 2006).

Lors d'expositions chroniques, la perturbation des macromolécules entraîne des effets indirects. Les changements structuraux du cytosquelette et les changements de fluidité de la bicouche phospholipidique altèrent la stabilité de la membrane, provoquant une perte de sa fonction, en altérant l'activité des protéines transmembranaires (Cossins, 1994; Hazel, 1995; Košťál, 2010). L'altération de structure des enzymes perturbe également l'homéostasie¹ de l'organisme (Hayward et al., 2014; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). Plusieurs revues complètes ce sont intéressées aux conséquences physiologiques des

¹ L'homéostasie est défini comme une stabilité dynamique des fonctions physiologiques de l'organisme (Bernard, 1865).

dégâts provoqués par les températures froides (Denlinger and Lee Jr, 2010; Hayward et al., 2014; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). Les sous-parties qui vont suivre ont pour but de résumer les principaux dégâts provoqués par le froid chez les insectes. Ces différents éléments sont également illustrés dans la Figure 7.

II.1.b.i. Perturbation de l'homéostasie cellulaire

En conditions non stressantes, les concentrations en ions de l'hémolymphe sont régulées par la sécrétion de fluides riches en ions K^+ dans les tubes de Malpighi et la réabsorption simultanée des ions Na^+ et de l'eau dans le proctodeum (Des Marteaux et al., 2017; Des Marteaux and Sinclair, 2016; Edney, 2012; Harrison et al., 2012; Maddrell and O'Donnell, 1992; Ramsay, 1954). L'altération de la fluidité membranaire provoquée par les diminutions de température altère la perméabilité ionique des membranes (Hazel, 1989¹), provoquant une perte de l'homéostasie ionique (Hazel, 1995; Košťál, 2010 ; Figure 7). Les activités des protéines transmembranaires (transporteurs, canaux ioniques...) peuvent être altérées par ces changements de fluidité (Cossins, 1994; Hazel, 1989; Illsley et al., 1987), mais également par l'effet cinétique des températures froides (Košťál et al., 2004; MacMillan and Sinclair, 2011a; Zachariassen et al., 2004 ; Figure 7). Ces perturbations participent à la dérégulation de l'équilibre entre les transports passifs et actifs des ions (Košťál et al., 2004, 2006; MacMillan et al., 2012b, 2015a; Overgaard and MacMillan, 2017 ; Figure 7). Le déséquilibre ionique se traduit généralement par une augmentation de la concentration en ion Na^+ dans la lumière du tube digestif ou d'autres organes. La forte pression osmotique provoquée par ces mouvements d'ions entraîne des mouvements d'eau vers les organes (Košťál et al., 2004; MacMillan and Sinclair, 2011b), et donc une plus faible teneur hydrique dans l'hémolymphe, provoquant une hyperkaliémie (forte concentration en ions K^+ , Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016; MacMillan et al., 2015b, 2016b ; Figure 7). Cette perte d'homéostasie hydrique et ionique provoque la dépolarisation des membranes cellulaires, induisant une perte du potentiel électrochimique de la membrane qui altère les potentiels d'actions des cellules musculaires et nerveuses (Figure 7). La rigidification et la dépolarisation des membranes induisent également un dysfonctionnement de l'activité des canaux Ca^{2+} des synapses qui contrôlent la libération des neurotransmetteurs (Findsen et al., 2016; Hazel, 1989; Rohrbough and Broadie, 2005). Une perte de la coordination au sein du système neuromusculaire résulte

¹ Note : bien que la plupart de ses travaux portent sur les poissons, les études de Hazel sur la fluidité membranaire chez les ectothermes sont une référence, et des mécanismes similaires se produisent chez les insectes.

de ces dérèglements, se traduisant par un coma (état défini par une absence totale, mais réversible, de mouvements causée par une exposition au froid ; Andersen et al., 2015; Armstrong et al., 2012; Hosler et al., 2000; Kelty et al., 1996; MacMillan et al., 2014; Overgaard and MacMillan, 2017; Robertson, 2004; Rodgers et al., 2007, 2010 ; Figure 7).

La perte d'homéostasie hydrique et ionique peut également endommager les cellules et les tissus (Košťál et al., 2004, 2006; MacMillan et al., 2014; MacMillan and Sinclair, 2011b) ; Figure 7). En effet, la dépolarisation des membranes est une étape d'initiation de l'apoptose (Bortner et al., 2001) et chez *D. melanogaster*, une exposition au froid provoque la mort cellulaire par apoptose dans les cellules des muscles thoraciques liés au vol (Yi et al., 2007).

Les températures froides provoquent également des désordres métaboliques, comme le ralentissement de la cinétique des réactions enzymatiques (Košťál et al., 2004; MacMillan and Sinclair, 2011a; Zachariassen et al., 2004 ; Figure 7). Plusieurs études ont également montré qu'après un stress thermique, la concentration en acides aminés libres augmente chez les insectes, y compris pour les acides aminés essentiels, qui ne peuvent pas être synthétisés *de novo*. (Colinet et al., 2012a; Košťál et al., 2011a; Lalouette et al., 2007; Overgaard et al., 2007). Cette accumulation stochastique d'acides aminés résulte très probablement de la dénaturation des protéines (Franks and Hatley, 1991; Todgham et al., 2007). Ces altérations entraînent un dysfonctionnement des voies métaboliques, provoquant une perte d'homéostasie (Colinet et al., 2012a; Overgaard et al., 2007; Teets et al., 2012; Williams et al., 2014 ; Figure 7). L'altération du métabolisme énergétique (glycolyse, cycle de Krebs) est également un des effets majeurs des températures froides (Michaud et al., 2008; Michaud and Denlinger, 2007, 2010; Overgaard et al., 2007). Les dysfonctionnements des voies métaboliques peuvent provoquer une accumulation de déchets métaboliques toxiques (Štětina et al., 2018), pouvant compromettre l'intégrité cellulaire (Figure 7).

Les dysfonctionnements du métabolisme entraînent également une surproduction de dérivés réactifs de l'oxygène (DRO) (Rojas and Leopold, 1996 ; Figure 7). Les DRO sont produits naturellement par le métabolisme aérobie, et sont naturellement éliminés par l'organisme, via l'action d'antioxydants (Livingstone, 2001). Toutefois lors d'un stress (ici un stress thermique), la production de DRO peut augmenter, provoquant une perturbation de l'équilibre entre la production de DRO et leur élimination par les antioxydants, induisant des dégâts oxydatifs (Lopez-Martinez et al., 2008). Le stress oxydatif induit des dégâts au niveau des protéines (dégradation des acides aminés, modifications de l'activité

enzymatique ; Stadtman and Levine, 2003), mais peut aussi dégrader les molécules d'ADN, en provoquant des mutations ou des délétions (Imlay, 2003).

La production de DRO et la perturbation de la fluidité des bicouches phospholipidiques pourraient également compromettre l'intégrité des mitochondries (Hazel, 1995 ; Figure 7), dont l'activité est réduite par les températures froides (Colinet et al., 2017b; Kukal et al., 1989). La mitochondrie étant le siège de la respiration cellulaire, l'altération de son activité provoque un déficit de production d'ATP (Colinet, 2011; Coulson et al., 1992; Dollo et al., 2010; MacMillan et al., 2012a), pouvant participer au déséquilibre de l'homéostasie métabolique (Figure 7).

II.1.b.ii. Dépolymérisation des filaments d'actine et de myosine

Les perturbations de l'activité musculaire dues aux températures froides (Findsen et al., 2014, 2016) pourraient également être liées à un dysfonctionnement des filaments d'actine et de myosine (Overgaard and MacMillan, 2017 ; Figure 7), comme c'est le cas lors d'un stress thermique chaud, qui provoque l'agrégation des filaments de myosine (Melkani et al., 2010).

En plus de l'altération de la fluidité de la bicouche lipidique des membranes, les températures froides altèrent la structure des cellules en agissant sur la conformation des polymères composant le cytosquelette, comme les filaments d'actine ou les microtubules (Cottam et al., 2006; Des Marteaux et al., 2018; Kayukawa and Ishikawa, 2009; Kim et al., 2006). Le cytosquelette garantit la structure et est essentiel au fonctionnement de la cellule (Amos and Amos, 1991). Les altérations du cytosquelette pourraient ainsi affecter les fonctions des tissus et des organes, augmentant la perméabilité intercellulaire (hydrique et ionique) des tissus par exemple (Des Marteaux et al., 2018; Kayukawa and Ishikawa, 2009 ; Figure 7).

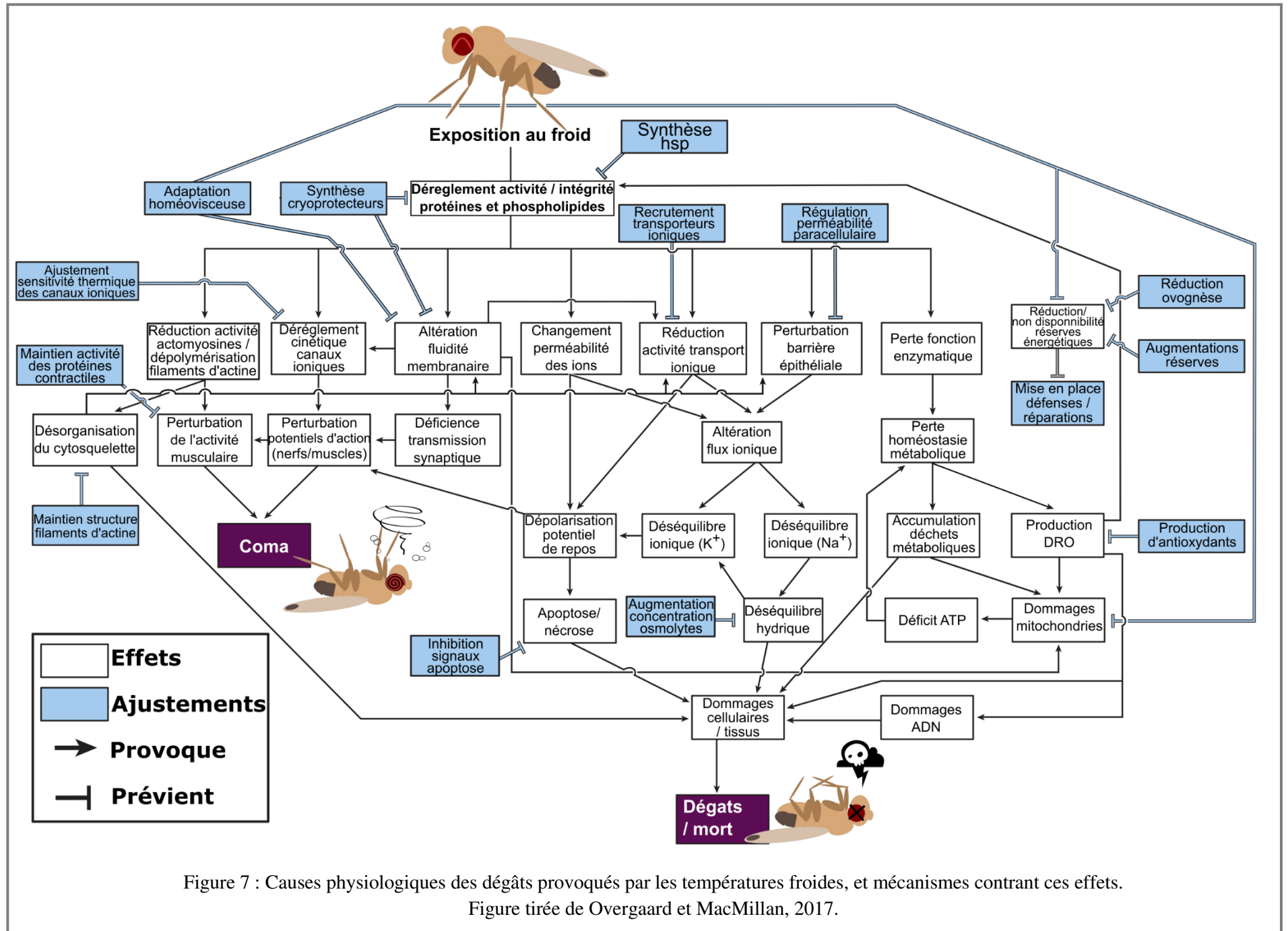


Figure 7 : Causes physiologiques des dégâts provoqués par les températures froides, et mécanismes contrant ces effets.
 Figure tirée de Overgaard et MacMillan, 2017.

II.2. Mécanismes sous-jacents à l'augmentation de la tolérance au froid

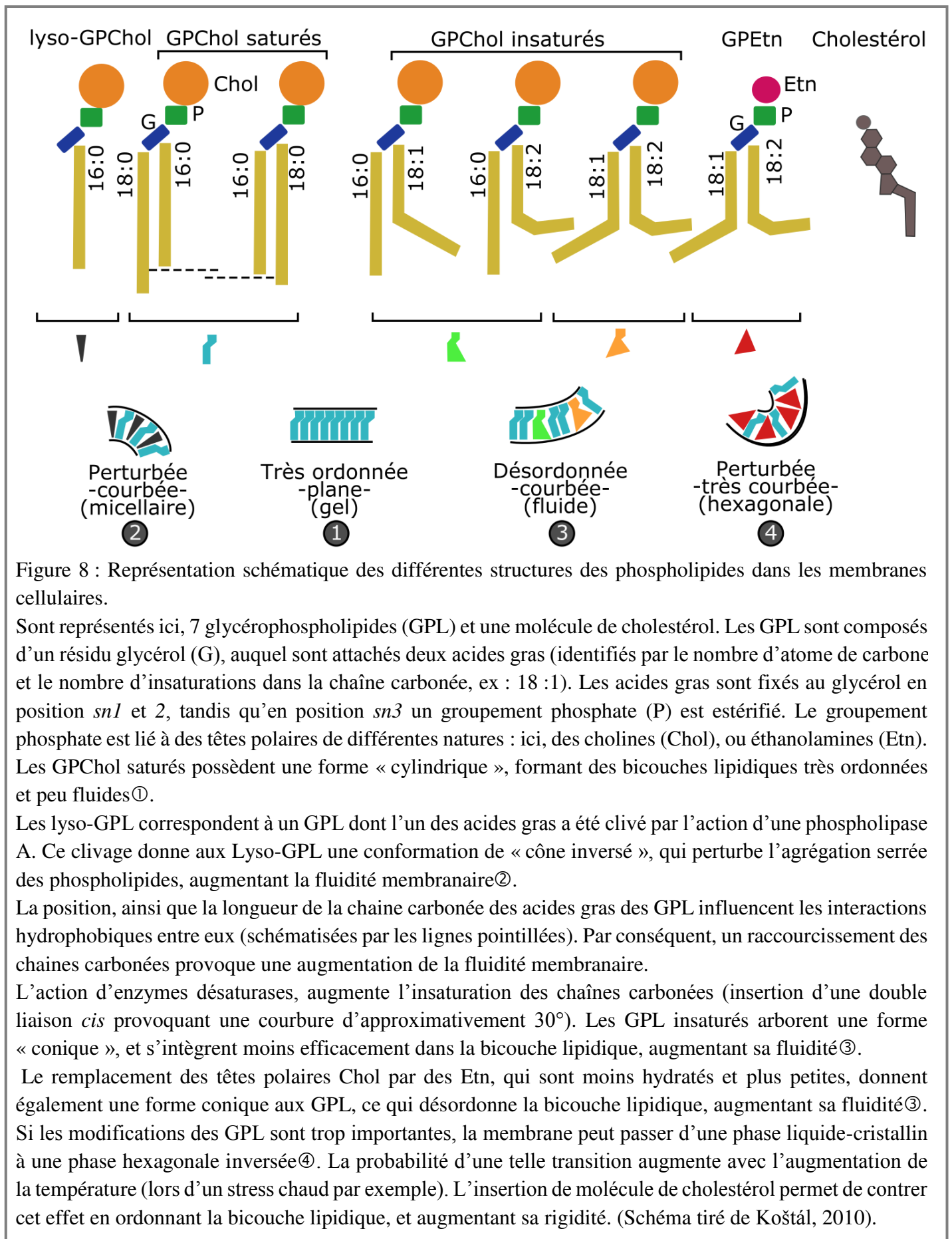
Pour faire face aux défis physiologiques provoqués par un stress froid modéré, les insectes possèdent un vaste arsenal d'ajustements. Un grand nombre de travaux ont revu ces processus en détail (Denlinger and Lee Jr, 2010; Hayward et al., 2014; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). Par conséquent les sous-parties qui vont suivre ont pour objectif de résumer les principaux ajustements liés à l'augmentation de la thermotolérance chez les insectes. Ces différents éléments sont illustrés dans la Figure 7.

II.2.a. Maintien de la structure des macromolécules

II.2.a.i. L'adaptation homéovisqueuse des membranes

Une des réponses physiologiques aux baisses de température la plus conservée parmi les ectothermes est l'adaptation homéovisqueuse des membranes cellulaires (Figure 7). L'adaptation homéovisqueuse regroupe les modifications de la composition de la bicouche de glycérophospholipides (GPL) qui permettent d'ajuster la fluidité de la membrane en fonction de la température (Hazel, 1995; Sinensky, 1974) afin de maintenir son bon fonctionnement lors d'expositions au froid (Košťál, 2010; Lee Jr et al., 2006). Des ajustements similaires peuvent se mettre en place pour éviter les transitions de phase de la membrane (voir Figure 6), plutôt que de maintenir sa fluidité. On parle alors d'adaptation homéophasique (Hazel and Williams, 1990).

Différentes restructurations des GPL permettent le maintien de la fluidité membranaire. Par exemple, l'augmentation de la proportion de Lyso-GPL au sein de la bicouche lipidique est connue comme jouant un rôle dans la réponse aux basses températures lors de fluctuations thermiques chez *D. melanogaster* (Colinet et al., 2016). Les changements de têtes polaires, classiquement la modification de la proportion de phosphoéthanolamines (GPEtn) par rapports aux phosphocholines (GPChol), est une réponse commune aux températures froides chez les insectes, que ce soit lors de la diapause, ou lors de l'acclimatation (Colinet et al., 2016; Cooper et al., 2012; Goto and Katagiri, 2011; Overgaard et al., 2008; Tomcala et al., 2006). La désaturation des chaînes carbonées des acides gras des GPL est un autre mécanisme de l'adaptation homéovisqueuse chez les insectes, en réponse à la diapause ou à l'acclimatation (Bashan and Cakmak, 2005; Kayukawa et al., 2007; Košťál and Simek, 1998). Toutefois cet ajustement ne semble pas être utilisé par les espèces du genre *Drosophila* (Colinet et al., 2016; Cooper et al., 2012; MacMillan et al., 2009; Ohtsu et al., 1999; Overgaard et al., 2008). Toujours au niveau des acides gras, l'augmentation de la tolérance au froid provoquée par les fluctuations



thermiques, l'adaptation, la diapause ou est souvent corrélée à un raccourcissement des chaînes carbonées (Bahrndorff et al., 2007; Bashan et al., 2002; Colinet et al., 2016; Michaud and Denlinger, 2006; Ohtsu et al., 1998, 1999; Overgaard et al., 2006).

Les mitochondries possédant également une bicouche lipidique, l'adaptation homéovisqueuse peut garantir leur intégrité lors d'exposition au froid, permettant de maintenir leur activité (Košťál, 2010); Figure 7). Les différents mécanismes permettant l'ajustement de la fluidité membranaire, sont présentés dans la Figure 8.

II.2.a.ii. Maintien de la structure du cytosquelette

Comme nous l'avons vu précédemment, la structure du cytosquelette est compromise par les températures froides. Son maintien est donc primordial pour garantir l'intégrité de la cellule. La stabilité des filaments d'actine est un facteur important lors des fluctuations thermiques, de la diapause, de l'acclimatation ou de l'acclimatation rapide (Colinet et al., 2012b; Gerken et al., 2015; Kim et al., 2006; Königer and Grath, 2018; Teets et al., 2012; Torson et al., 2015). Cette stabilisation est régulée par une série de phosphorylations des composants du cytosquelette (Colinet et al., 2017a).

II.2.a.iii. Synthèse de cryoprotecteurs

Les cryoprotecteurs sont des métabolites de faible poids moléculaire dont l'accumulation permet une augmentation de la tolérance au froid ou au gel (Lee Jr, 2010). La diapause, l'acclimatation, l'acclimatation rapide et l'adaptation au froid induisent une accumulation de cryoprotecteurs chez les insectes. Les polyols, et particulièrement le glycérol, sont les cryoprotecteurs les plus communs en réponse à l'augmentation de la thermotolérance (Lee Jr, 2010; Salt, 1961; Sømme, 1982; Storey and Storey, 2005; Vesala et al., 2012). D'autres molécules possèdent des qualités cryoprotectrices et sont accumulés lors de l'augmentation de la tolérance au froid, comme les sucres simples (fructose, glucose, maltose, sucrose, tréhalose... ; Baust and Edwards, 1979; Colinet et al., 2012a; Ditrich and Košťál, 2011; Fields et al., 1998; Kimura, 1982; Košťál et al., 2011a; Overgaard et al., 2007; Zeng et al., 2008), ou les acides aminés libres (Valine, Leucine, Serine, Thréonine, Isoleucine, Proline, Histidine, Alanine... ; Colinet et al., 2012a; Fields et al., 1998; Foray et al., 2013; Hanzal and Jegorov, 1991; Košťál et al., 2011a; Li et al., 2015; Michaud and Denlinger, 2007; Morgan and Chippendale, 1983; Storey, 1983). Concernant les acides aminés, plusieurs travaux ont démontré expérimentalement que l'augmentation artificielle de la concentration d'acides aminés, et notamment la proline, provoquait une forte hausse de la thermotolérance des insectes

(Košťál et al., 2016a), permettant aux larves de *Drosophila melanogaster* de tolérer la formation de gel interne (Košťál et al., 2012), et les larves de la mouche *Chymomyza costata* de survivre à un bain d'azote liquide (Košťál et al., 2011c).

Certains insectes sont capables d'accumuler des concentrations de cryoprotecteurs extrêmement importantes, comme par exemple la larve en diapause de la guêpe parasitoïde *Bracon cephi*, qui est capable d'accumuler 5 M de glycérol, soit environ 25% de sa masse ! (Salt, 1961). A de telles concentrations, ces molécules permettent de diminuer la température de congélation de l'hémolymphe, empêchant la formation de gel interne par leurs propriétés osmotiques ou colligatives (Crosthwaite et al., 2011; Storey and Storey, 1991; Zachariassen, 1985). Toutefois de nombreuses études ont montré que l'accumulation de cryoprotecteurs à des quantités beaucoup plus faibles (de l'ordre du μM ou mM) permettait d'augmenter la thermotolérance des insectes (Colinet et al., 2012a; Košťál et al., 2011a). A faible concentration, il est supposé que les cryoprotecteurs aident à maintenir la stabilité de la structure des membranes cellulaires et des protéines (Arakawa and Timasheff, 1982; Cacela and Hinch, 2006; Carpenter and Crowe, 1988; Crowe et al., 1988; Yancey, 2005); Figure 7), potentiellement par exclusion préférentielle des solutés (Gekko and Timasheff, 1981; Kim et al., 2018b; Košťál et al., 2016a).

II.2.a.iv. Synthèse de protéines chaperonnes

Une des réponses à un stress thermique commune à la majeure partie des organismes est la synthèse de protéines de choc thermique (PCT). Les PCT sont des protéines chaperonnes, exprimées de manière constitutive chez les organismes, veillant au bon fonctionnement de la machinerie cellulaire en facilitant le repliement des polypeptides nouvellement synthétisés (Feder and Hofmann, 1999; Kültz, 2003). Les PCT permettent le transport, le maintien de la conformation, l'assemblage des protéines et dirigent la dégradation des protéines potentiellement dénaturées (Feder and Hofmann, 1999; Parsell and Lindquist, 1993; Sørensen et al., 2003). Leur expression est toutefois amplifiée lors de la réponse à une grande variété de stress (Feder and Hofmann, 1999), notamment le stress thermique froid (Burton et al., 1988; Colinet et al., 2010b ; Figure 7), où leur expression est essentielle à la récupération après le stress (Colinet et al., 2010a; Rinehart et al., 2007).

II.2.b. Maintien de l'homéostasie cellulaire

II.2.b.i. Homéostasie ionique et hydrique

Nous avons vu précédemment que les stress froids provoquent des perturbations importantes de l'équilibre hydrique et ionique (voir **partie II.1.b.i**). Par conséquent, l'augmentation de la thermotolérance des insectes est souvent corrélée avec un maintien de l'homéostasie hydrique et ionique (Des Marteaux et al., 2017; Des Marteaux and Sinclair, 2016; Košťál et al., 2004; MacMillan et al., 2015a).

Chez les insectes, l'augmentation de la tolérance au froid induite par la diapause et l'acclimatation est liée avec la préservation de l'équilibre ionique de part et d'autre des membranes cellulaires (Armstrong et al., 2012; Coello Alvarado et al., 2015; Košťál et al., 2004, 2006 ; Figure 7). Ce maintien de l'homéostasie ionique a également été identifiée chez l'espèce modèle *Drosophila melanogaster* (Armstrong et al., 2012; MacMillan et al., 2015a, 2015c). Les principaux mécanismes sous-jacents au maintien de l'équilibre ionique sont la plasticité de la sensibilité thermique des canaux ioniques, ainsi que l'augmentation de l'activité des transporteurs ioniques (Armstrong et al., 2012; Findsen et al., 2016; Frolov and Singh, 2013; MacMillan et al., 2015c; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017 ; Figure 7). Les jonctions cellulaires contrôlent la diffusion intercellulaire des fluides et des ions (Beyenbach and Piermarini, 2011; Jonusaite et al., 2016), par conséquent le maintien de leur structure pourrait également être un des mécanismes permettant de garantir l'homéostasie ionique et hydrique (Overgaard and MacMillan, 2017 ; Figure 7). Afin de compenser la réduction progressive des concentrations en Na⁺ dans l'hémolymphe due aux températures froides, les insectes acclimatés ou adaptés au froid sont capables d'augmenter la concentration d'autres osmolytes compatibles (tels que les cryoprotecteurs, décrits dans la **partie II.2.a.iii**, qui jouent ici un rôle osmoprotecteur) ce qui a pour effet de préserver l'équilibre osmotique et d'éviter une perte de l'homéostasie hydrique (MacMillan et al., 2015b; Olsson et al., 2016 ; Figure 7).

La dépolarisation des membranes et les perturbations de la concentration en ions Ca²⁺ provoquées par le froid peuvent induire des effets cascades menant à l'apoptose (Bortner et al., 2001; Yi et al., 2007 ; voir **partie II.1.b.i**). La concentration en ions Ca²⁺ joue également un rôle important dans l'initiation de l'acclimatation rapide (Bortner et al., 2001; Teets et al., 2008, 2013), qui est par ailleurs capable d'inhiber l'apoptose en bloquant l'activité des caspases (Yi et al., 2007; Yi and Lee, 2011 ; Figure 7).

La régulation de l'équilibre hydrique et ionique permet de préserver les potentiels électrochimiques des membranes, garantissant ainsi l'intégrité des fonctions neuromusculaires (Armstrong et al., 2012; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017); Figure 7). De plus, l'acclimatation chez les insectes provoque la surexpression de protéines ou de transcrits liés à la fonction des protéines contractiles (actine, myosine, flightine... ; Colinet et al., 2013; Finsen et al., 2016; MacMillan et al., 2016a; Parker et al., 2015), qui pourraient également assurer leur activité musculaire (Figure 7).

II.2.b.ii. Homéostasie métabolique

Malmendal et al. (2006) ont démontré qu'un stress chaud provoquait une perte de l'homéostasie métabolique chez des drosophiles (*D. melanogaster*) non acclimatées, alors que l'homéostasie était maintenue chez les drosophiles acclimatées. Des études supplémentaires ont montré une perte d'homéostasie similaire après un stress froid, compensée chez les drosophiles tolérantes au froid (acclimatées ou sélectionnées ; Colinet et al., 2012a; Overgaard et al., 2007; Williams et al., 2014).

Les mécanismes sous-jacents au maintien de l'homéostasie métabolique sont très complexes et pourraient découler du maintien de la structure des macromolécules (membranes, protéines... ; Figure 7), et des réajustements de l'activité des voies du métabolisme central (Colinet et al., 2007; Li et al., 2015; Lu et al., 2014; MacMillan et al., 2016a ; Figure 7). La diapause, l'acclimatation ou l'adaptation au froid permettent également aux insectes d'ajuster leurs défenses antioxydantes, afin de compenser le déséquilibre entre production et suppression de DRO liés avec la perte d'homéostasie, afin de prévenir les dommages liés au stress oxydatif (Blagojević, 2007; Grubor-Lajsic et al., 1997; Jovanović-Galović et al., 2004; Stanic et al., 2004 ; Figure 7).

II.2.c. L'importance des réserves énergétiques

Les réserves énergétiques des insectes jouent un rôle prépondérant dans la survie lors d'expositions aux températures froides (Hahn and Denlinger, 2007, 2011; Sinclair, 2015; Sinclair and Marshall, 2018). Ces réserves fournissent l'énergie nécessaire à l'organisme pour mettre en place les ajustements physiologiques permettant de moduler sa tolérance au froid, réparer les dégâts provoqués par le stress thermique et survivre à la période hivernale où la disponibilité en ressources est limitée.

Lors d'un stress froid, les insectes sont souvent dans l'incapacité de s'alimenter, les conséquences des expositions prolongées au froid sont donc généralement dues aux effets

combinés du froid et du jeûne qui y est associé. En effet, l'exposition aux basses températures induit chez les insectes un jeûne contraint (lorsque l'insecte est en coma) ou délibéré (lors d'une diapause prolongée par exemple). A terme, de longues périodes d'expositions peuvent provoquer l'épuisement de ces réserves et donc la mort de l'individu (Renault et al., 2002). Si l'insecte a la possibilité de s'alimenter pendant le stress thermique, les réserves lipidiques sont préservées et sa probabilité de survie est donc améliorée (Košťál et al., 2016b), soulignant l'importance de ces réserves dans la tolérance au froid des insectes.

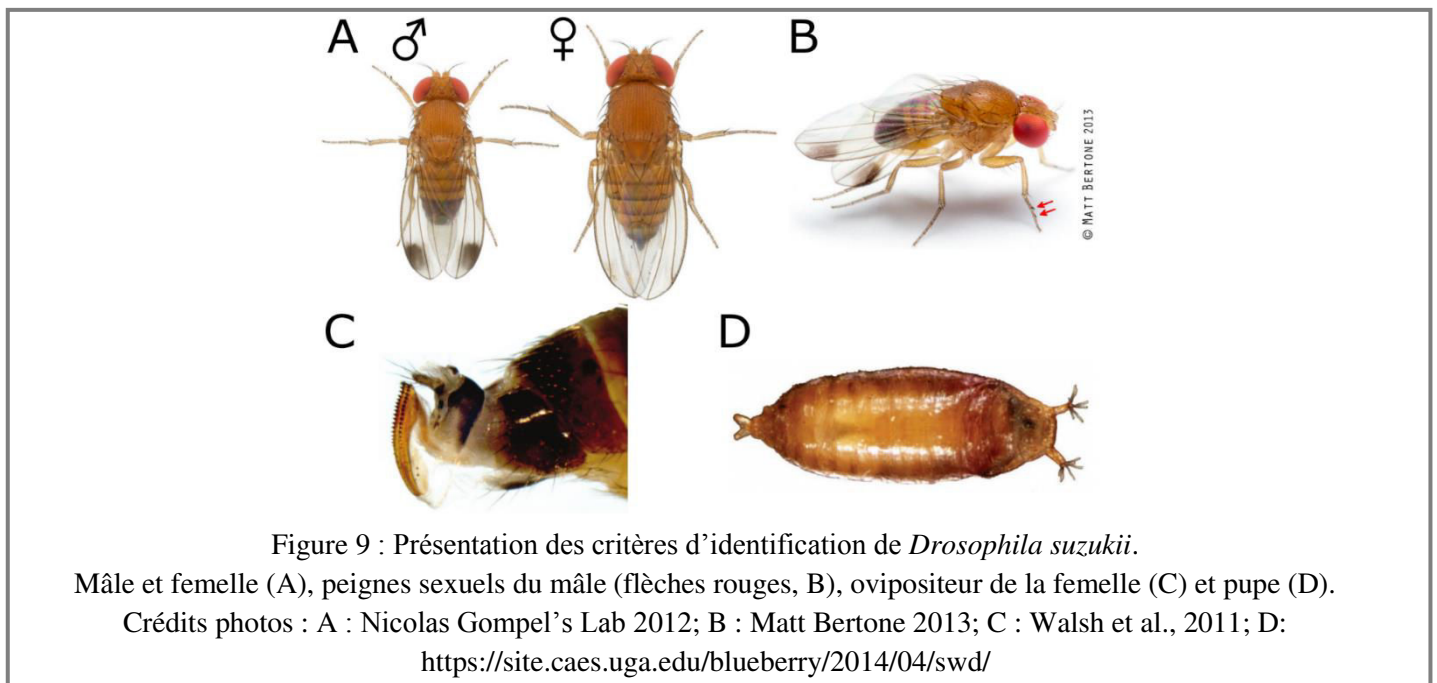
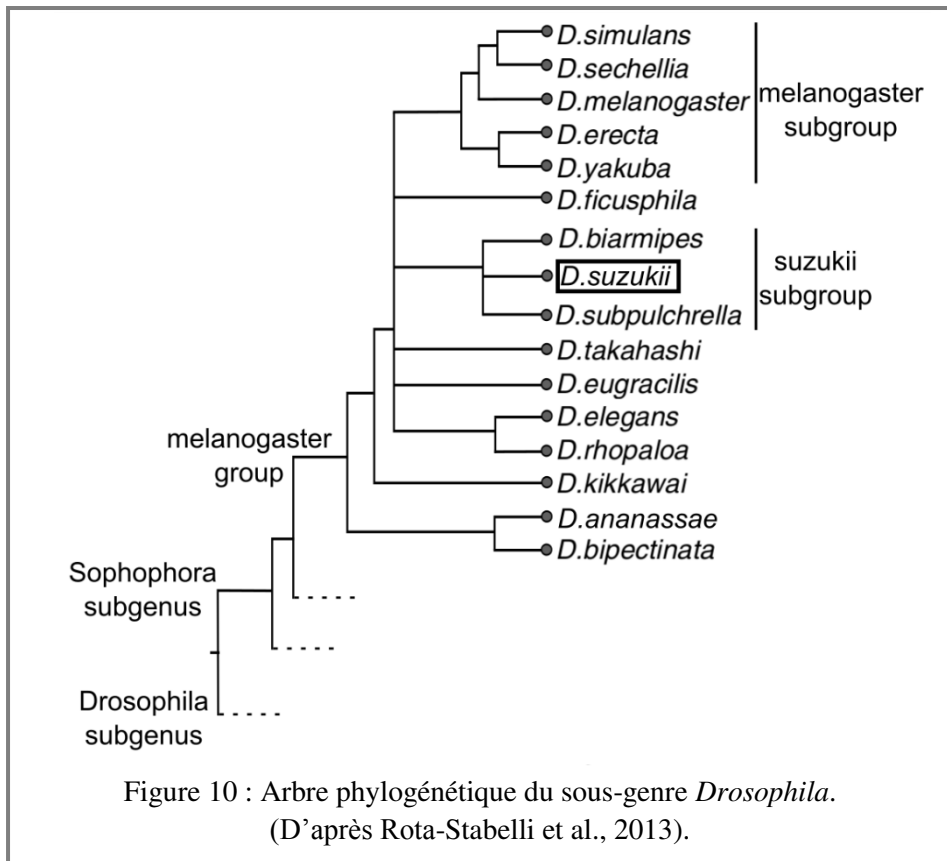
Les triacylglycerides (TAG) représentent la majeure partie des réserves énergétiques chez les insectes, et sont donc impliqués dans la survie hivernale (Hahn and Denlinger, 2011; Ohtsu et al., 1993; Sinclair and Marshall, 2018). Toutefois la diminution des températures tend à rigidifier les TAG, compromettant leur disponibilité et leur utilisation par le métabolisme des insectes. Lors de processus d'acclimatation, d'adaptation au froid ou de diapause, des ajustements similaires à l'adaptation homéovisqueuse des membranes se mettent en place au sein des TAG : augmentation du degré d'insaturation des acides gras, et raccourcissement des chaînes carbonées (Košťál and Simek, 1998; Ohtsu et al., 1993; Van Dooremalen and Ellers, 2010). Ces ajustements permettent d'abaisser le point de fusion de ces réserves énergétiques, augmentant leur fluidité aux basses températures et garantissant ainsi leur disponibilité (Košťál and Simek, 1998; Rozsypal et al., 2014; Van Dooremalen and Ellers, 2010 ; Figure 7).

L'arrêt de la fonction de reproduction est une réponse classique aux baisses de la température chez les insectes, comme c'est le cas lors de la diapause (Košťál, 2006). L'arrêt de la fonction de reproduction pourrait faire partie d'un mécanisme propre à l'ajustement de la thermotolérance : l'énergie non allouée à la production de gamètes pourrait être utilisée dans la mise en place des systèmes de défenses ou de réparation suite aux stress thermiques (Lirakis et al., 2018; Marshall and Sinclair, 2010; Mensch et al., 2017 ; Figure 7).

III. *Drosophila suzukii*, présentation de l'espèce

III.1. Taxonomie et identification

Drosophila suzukii (Matsumura, 1931), communément appelée « drosophile à ailes tachetées », est un diptère du genre *Drosophila*, (sous-genre *Sophophora*), appartenant au groupe *melanogaster*, sous-groupe *suzukii* (Linde and Houle, 2008; Ometto et al., 2013; Rota-Stabelli et al., 2013; Yang et al., 2012). Par conséquent cette espèce est phylogénétiquement proche de l'espèce modèle *Drosophila melanogaster* (Figure 10).



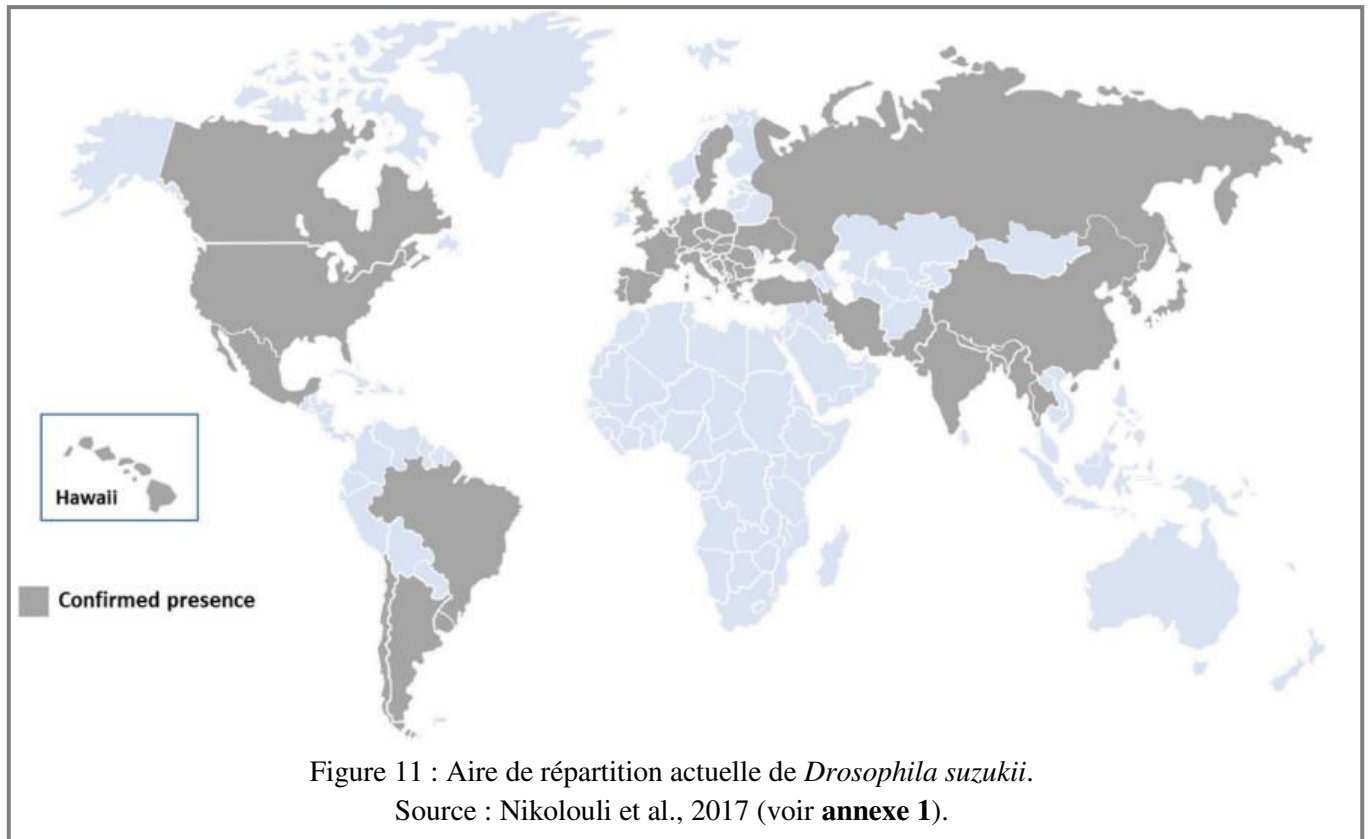
Drosophila suzukii est un insecte de petite taille (3 – 4 mm), de couleur brun clair (Kanzawa, 1939; Walsh et al., 2011). Il existe chez cette espèce un dimorphisme sexuel important : les femelles sont généralement de plus grande taille que les mâles. Ces derniers possèdent un point noir sur la partie terminale de chaque aile (Figure 9A ; Rota-Stabelli et al., 2013). Cette caractéristique permet de différencier *D. suzukii* des autres espèces de drosophiles que l'on trouve sous nos latitudes. La présence de deux peignes sexuels sur les premiers tarses des pattes antérieures des mâles permet également de les différencier d'autres espèces de drosophiles (Figure 9B ; Hauser, 2011). Les femelles sont aisément discernables des femelles d'autres espèces grâce à leur ovipositeur sclérotisé de taille imposante (Figure 9C ; Hauser, 2011). Enfin, la morphologie en forme d'étoile des tubes respiratoires des pupes constitue également un critère d'identification de *D. suzukii* (Figure 9D ; Okada, 1968).

III.2. Aire de répartition actuelle

Drosophila suzukii possède une aire de répartition extrêmement large qui pourrait encore s'étendre (Gibert et al., 2016; Ørsted and Ørsted, 2019; Santos et al., 2017). Cette drosophile est originaire d'Asie de l'Est (Kanzawa, 1939 ; Asplen et al., 2015 ont dressé une revue détaillée de son aire de répartition dans cette région) et proviendrait à l'origine de zones de climat tempéré, tels que des forêts montagneuses (Ometto et al., 2013). Elle a été reportée en tant qu'espèce invasive à Hawaï dès 1980 (Kaneshiro, 1983) puis a été identifiée aux Etats Unis en 2008 (Hauser et al., 2009), au Canada en 2009 (Hauser, 2011) ainsi qu'en Amérique du Sud, notamment au Brésil et en Uruguay en 2013, (Deprá et al., 2014; González et al., 2015) ainsi qu'en Argentine, au Mexique et au Chili en 2014 (Lasa and Tadeo, 2015; Lue et al., 2017; Medina-Muñoz et al., 2015).

En Europe, *D. suzukii* a été recensée pour la première fois en 2008 en Espagne et en Italie (Calabria et al., 2012; Raspi et al., 2011), puis en France en 2009 (Weydert and Bourgouin, 2012). Cependant, il semblerait que la zone d'origine de son invasion soit située dans le sud de la France, dans les environs d'Avignon (Cini et al., 2014). Par la suite, *D. suzukii* a été recensée en 2011 en Suisse (Baroffio and Fisher, 2011), en Slovénie (Seljak, 2011), en Croatie (Masten Milek et al., 2011), en Autriche (Lethmayer, 2011), en Allemagne (Vogt et al., 2012) et en Belgique (Mortelmans et al., 2012). Elle a depuis continué son expansion, puisqu'elle a été identifiée aux Pays-Bas, en Angleterre et en Hongrie en 2012 (Cuthbertson et al., 2014; Lengyel et al., 2015; Panel et al., 2018), au Monténégro et en Roumanie en 2013 (Chireceanu et al., 2015; Radonjic and Hrcic, 2015) puis en Suède (Manduric, 2017), en Pologne (Łabanowska

and Piotrowski, 2015), en Turquie (Orhan et al., 2016), en Serbie (Toševski et al., 2014) et en Ukraine (Lavrinenko et al., 2017) en 2014.



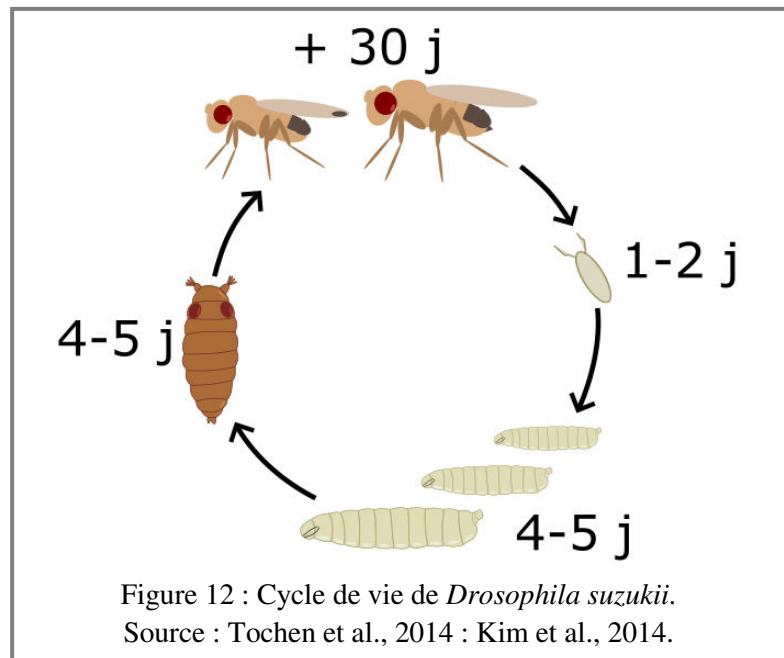
Cet historique met en évidence la rapidité et l'efficacité de l'invasion de *D. suzukii*, qui en seulement une dizaine d'année s'est implantée dans une vingtaine de pays répartis sur plusieurs continents. Son expansion rapide est premièrement due à une forte capacité de dispersion : c'est une espèce migratrice dans son aire d'origine (Mitsui et al., 2010; Rota-Stabelli et al., 2013), et le commerce international des fruits lui permet d'atteindre rapidement de nouveaux habitats (Cini et al., 2014; Rota-Stabelli et al., 2013). Plusieurs travaux ont également estimé que *D. suzukii* est capable de voler sur de longues distances (Tait et al., 2018; Wong et al., 2018). Cette drosophile profite également d'autres adaptations, contribuant grandement au succès de son invasion, qui sont présentées dans les paragraphes suivants.

III.3. Les paramètres du succès de son invasion

III.3.a. Cycle de vie

Les diptères étant holométaboles, *Drosophila suzukii* possède un cycle de vie divisé en quatre stades (Figure 12) : le stade œuf, durant approximativement 48 heures, le stade larvaire (divisé en 3 stades : L1, L2, L3) durant 4,5 jours en moyenne, le stade pupes durant également 4,5 jours en moyenne, et enfin le stade imaginal, pour une durée totale de l'œuf à l'adulte de 11

jours en moyenne à 25 °C. L'adulte peut survivre plus de 30 jours en conditions de laboratoire (Kanzawa, 1939; Kim et al., 2015; Tochen et al., 2014). *Drosophila suzukii* est une espèce multivoltine, son temps de génération court lui permet de réaliser jusqu'à plus de 10 générations par an (Mitsui et al., 2010; Tochen et al., 2014; Walsh et al., 2011).



Les études quantifiant la fécondité de cette espèce montrent des résultats variables en fonction des lignées et des milieux de pontes utilisés, mais de manière générale les femelles *D. suzukii* pondent une dizaine d'œufs par jour (Emiljanowicz et al., 2014; Kanzawa, 1939; Kim et al., 2015; Mitsui et al., 2006; Tochen et al., 2014). Au cours de sa vie, une femelle pourrait pondre de 200 à 630 œufs au total (Emiljanowicz et al., 2014; Kanzawa, 1939).

Cette forte fécondité, couplé à un temps de génération court, aident très probablement *D. suzukii* à générer des populations importantes, rapidement après son introduction dans un nouveau milieu (Rota-Stabelli et al., 2013).

III.3.b. Hôtes et compétition interspécifique

Les femelles *D. suzukii* pondent exclusivement leurs œufs dans les fruits sains (Hauser et al., 2009; Kanzawa, 1939; Lee et al., 2011; Mitsui et al., 2006). Leur ovipositeur sclérotisé (voir Figure 9C), leur permet de percer la peau des fruits mûrs et d'y déposer leurs œufs (Hauser et al., 2009). Cette spécialisation pour les fruits frais permet à *D. suzukii* d'éviter la compétition avec d'autres espèces de drosophiles qui s'attaquent généralement aux fruits en décomposition. De plus, le système agricole Européen comporte très peu de compétiteurs pour les fruits frais,

ce qui laisse le champ libre au développement des larves de *D. suzukii* (Rota-Stabelli et al., 2013).

Drosophila suzukii est également une espèce polyphage : elle peut se développer sur un vaste panel de plantes hôtes, aussi bien des plantes sauvages tel le gui (*Viscum album*) que des plantes cultivées, comme les fraises, les framboises, les mûres, les cerises, les abricots, les pêches, les prunes, les myrtilles, le raisin... (Cini et al., 2012; Kenis et al., 2016; Poyet et al., 2015). Les larves vont se nourrir de la chair du fruit et causer d'importants dégâts. Des pathogènes secondaires (bactéries, champignons) peuvent profiter de ces ouvertures et participer à la dégradation du fruit (Cini et al., 2012). Ce mode de reproduction provoque de forts dégâts sur les cultures ayant en conséquence un coût financier important. En effet, en 2011 les pertes économiques sur les fruits cultivés lui étant attribuées étaient estimés à trois millions d'Euros (Bolda et al., 2010; De Ros et al., 2015; Goodhue et al., 2011).

III.3.c. Faible pression de prédation dans les zones envahies

Les études sur les prédateurs naturels de *D. suzukii* sont majoritairement concentrées sur les parasitoïdes. Dans son aire d'origine *D. suzukii* est prédatée par plusieurs espèces de parasitoïdes, ciblant majoritairement les pupes (Kasuya et al., 2013; Kimura and Novković, 2015; Mitsui et al., 2007; Mitsui and Kimura, 2010; Nomano et al., 2015). Dans les aires envahies, peu de parasitoïdes parviennent à parasiter *D. suzukii*, que ce soit en Europe (Gabarra et al., 2015; Rossi Stacconi et al., 2013, 2015), en Amérique du Sud (Cancino et al., 2015; Moreno et al., 2015) ou en Amérique du Nord (Rossi Stacconi et al., 2013, 2015). Ces parasitoïdes sont majoritairement généralistes et parasitent une très large gamme d'hôtes (Chabert et al., 2012). Le faible taux de parasitisme de *D. suzukii* peut s'expliquer par le fait que cette espèce possède un système d'encapsulation des œufs de parasitoïdes très efficace (Chabert et al., 2012; Kacsoh and Schlenke, 2012; Poyet et al., 2013).

III.4. La thermotolérance de *D. suzukii* favorise-t-elle son invasion ?

III.4.a. Thermotolérance basale

Le fait que *D. suzukii* ait réussi à s'implanter en Europe et en Amérique du Nord montre qu'elle est capable de survivre aux températures hivernales de ces régions. Cette drosophile reste active en automne, où elle parasite les fruits disponibles (Poyet et al., 2015), puis passe l'hiver au stade adulte (Briem et al., 2018; Panel et al., 2018; Thistlewood et al., 2018). Néanmoins, les populations sont grandement réduites pendant l'hiver (Arnó et al., 2016;

Mazzetto et al., 2015; Wang et al., 2016), ce qui suggère que *D. suzukii* est peu tolérante au froid.

En effet, plusieurs expérimentations ont montré que la survie de *D. suzukii* est réduite lorsqu'elle est exposée à des températures froides (Dalton et al., 2011; Jakobs et al., 2015; Kimura, 2004; Plantamp et al., 2016; Ryan et al., 2016). Comme la plupart des autres espèces de drosophiles (Czajka and Lee, 1990), *D. suzukii* est une espèce intolérante au froid (voir **partie I.5**) : les individus succombent avant la formation de gel interne (Jakobs et al., 2015). Sa survie décroît rapidement lors de stress aigus (85% de mortalité après une heure à -7,2°C ; Jakobs et al., 2015), ou chroniques (50% de mortalité après 3j à 3°C ; Dalton et al., 2011; voir également Plantamp et al., 2016 et Ryan et al., 2016).

Ma thèse ayant débuté en 2016, peu d'informations concernant la thermobiologie de *D. suzukii* étaient alors disponibles. En effet, mis à part les études de Kanzawa (1939) et de Kimura (2004), la totalité des études portant sur la thermotolérance de cette espèce ont été réalisées lors des huit dernières années, dont la majeure partie depuis 2016. La plupart de ces études ont soit utilisé une seule température avec différentes durées d'exposition (Jakobs et al., 2015), soit un gradient de températures mais avec une seule durée d'exposition (Kimura, 2004). La tolérance au froid étant une réponse décrite selon deux dimensions : la durée d'exposition et l'intensité du froid ; analyser l'un ou l'autre de ces paramètres semble insuffisant (Rezende et al., 2014). Il était donc nécessaire de mettre en place des plans expérimentaux permettant d'établir avec plus de précision la thermotolérance basale de *D. suzukii*.

Il est également intéressant de noter qu'avant 2016, seul Kimura (2004) s'était intéressé à la tolérance au chaud de *D. suzukii*. Cet auteur a montré qu'une exposition de 24h à 32°C provoquait 50% de mortalité parmi les individus testés. Par la suite Eben et al. (2018) et Evans et al. (2018) ont montré que la survie de *D. suzukii* était fortement impactée lors d'expositions à des températures supérieures à 33°C. Durant l'été sous nos latitudes, les populations de *D. suzukii* subissent une forte diminution de leur densité (Pelton et al., 2016; Thistlewood et al., 2018), qui pourrait être due à une mortalité induite par les températures chaudes. Des informations supplémentaires concernant les températures maximales que cette espèce peut supporter sont donc requises afin de mieux prédire l'évolution des populations durant l'été.

III.4.b. Thermotolérance induite

En hiver, les adultes *D. suzukii* seraient capables de s'abriter dans des habitations ou autres constructions (Kanzawa, 1939; Kimura, 2004), ou de migrer vers des refuges aux

conditions climatiques tamponnées, comme des zones boisées (Cahenzli et al., 2018; Kimura, 2004; Santoiemma et al., 2018; Stockton et al., 2019; Thistlewood et al., 2018; Tonina et al., 2016; Wallingford et al., 2018; Zerulla et al., 2015). En effet, en période hivernale, des adultes sont capturés en forêt (Briem et al., 2016; Pelton et al., 2016). L'utilisation de ces zones refuges pourrait faciliter la survie hivernale de *D. suzukii* dans les régions envahies.

En plus des ajustements comportementaux, la plasticité de la tolérance au froid joue un rôle primordial dans la répartition des espèces, et dans le succès des invasions biologiques (voir **partie I.8**). Différentes études ont montré que la tolérance de *D. suzukii* était fortement plastique (Hamby et al., 2016). Plusieurs formes d'acclimations ont un effet positif sur sa thermotolérance, telles que l'acclimation rapide (Everman et al., 2018; Toxopeus et al., 2016), l'acclimation graduelle au stade adulte (Jakobs et al., 2015; Wallingford et al., 2016) ou l'acclimation développementale (Shearer et al., 2016; Toxopeus et al., 2016; Wallingford and Loeb, 2016). Elle est également capable de survivre durant plusieurs jours à -7.5°C après acclimation dynamique (refroidissement graduel ; Stockton et al., 2018). Cette plasticité importante contribuerait probablement à sa survie hivernale (Stockton et al., 2018).

Les adultes *D. suzukii* capturés durant l'hiver montrent un phénotype différent des individus capturés en période estivale (Guédot et al., 2018; Kanzawa, 1939; Panel et al., 2018; Shearer et al., 2016). Ce phénotype est caractérisé par une plus grande taille, une mélanisation plus importante de la cuticule, ainsi qu'un arrêt de la production d'œufs chez les femelles (Jakobs et al., 2015; Shearer et al., 2016; Wallingford et al., 2016; Zhai et al., 2016). Ce phénotype est inductible en laboratoire lorsque les individus sont placés à des températures froides ($< 12^{\circ}\text{C}$) avec une photopériode courte durant leur développement du stade œuf jusqu'à l'émergence des adultes (Shearer et al., 2016; Stephens et al., 2015; Toxopeus et al., 2016; Wallingford and Loeb, 2016). Ces drosophiles montrent alors une tolérance au froid largement accrue, pouvant survivre à des expositions à -10°C (Shearer et al., 2016; Stephens et al., 2015; Toxopeus et al., 2016; Wallingford et al., 2016; Wallingford and Loeb, 2016).

Les études portant sur la tolérance au froid de *D. suzukii* ont jusqu'alors évalué la survie de *D. suzukii* suite à des expositions à des températures constantes. Seule une récente étude a montré que l'utilisation de températures fluctuantes permettait d'augmenter la survie de *D. suzukii* en comparaison aux températures constantes (Grumiaux et al., 2019). Les réponses des insectes aux températures froides peuvent drastiquement varier lors d'expositions à des températures fluctuantes (*i.e.* RTF, voir **partie I.7.d**), cet aspect mérite donc d'être développé

chez *D. suzukii*. De plus, la très grande majorité des travaux traitant de la plasticité phénotypique de *D. suzukii* ont été produits depuis 2016. Dans cette période, une seule étude (Shearer et al., 2016) s'est intéressée aux mécanismes sous-jacents à la plasticité de la tolérance au froid chez *D. suzukii*, qui sont donc encore peu décrits.

IV. Objectifs et hypothèses de ma thèse

Une forte capacité de dispersion, un cycle de vie court, une grande variété d'hôtes, peu de compétiteurs pour les ressources et une faible pression de prédation sont autant de facteurs contribuant au succès de l'invasion de *D. suzukii*. La thermotolérance est également un facteur à ne pas négliger dans le succès des invasions biologiques. En effet, la température est un paramètre important, définissant la niche fondamentale des insectes, et influençant largement leur biologie (voir **partie I.2**). De plus, la plasticité de la thermotolérance des insectes invasifs facilite grandement le succès de leurs invasions (Bates et al., 2013; Kolar and Lodge, 2002; Zerebecki and Sorte, 2011 ; voir **partie I.8**). Toutefois, il apparaît après analyse de la littérature que la tolérance au froid de *D. suzukii* était peu connue avant 2016 et les études sur la plasticité de sa thermotolérance étaient alors quasi inexistantes. Les mécanismes physiologiques sous-jacents à la plasticité de la thermotolérance n'étant pas clairement décrits chez cette espèce, les stratégies qu'elle met en place durant l'hiver dans les régions envahies étaient donc encore inconnues. Il était donc nécessaire d'acquérir des connaissances fondamentales sur la thermobiologie de *D. suzukii* qui permettraient de comprendre ses capacités d'hivernage, et estimer l'évolution de son aire de répartition, afin d'adapter les stratégies de lutte biologique (Asplen et al., 2015; Hamby et al., 2016).

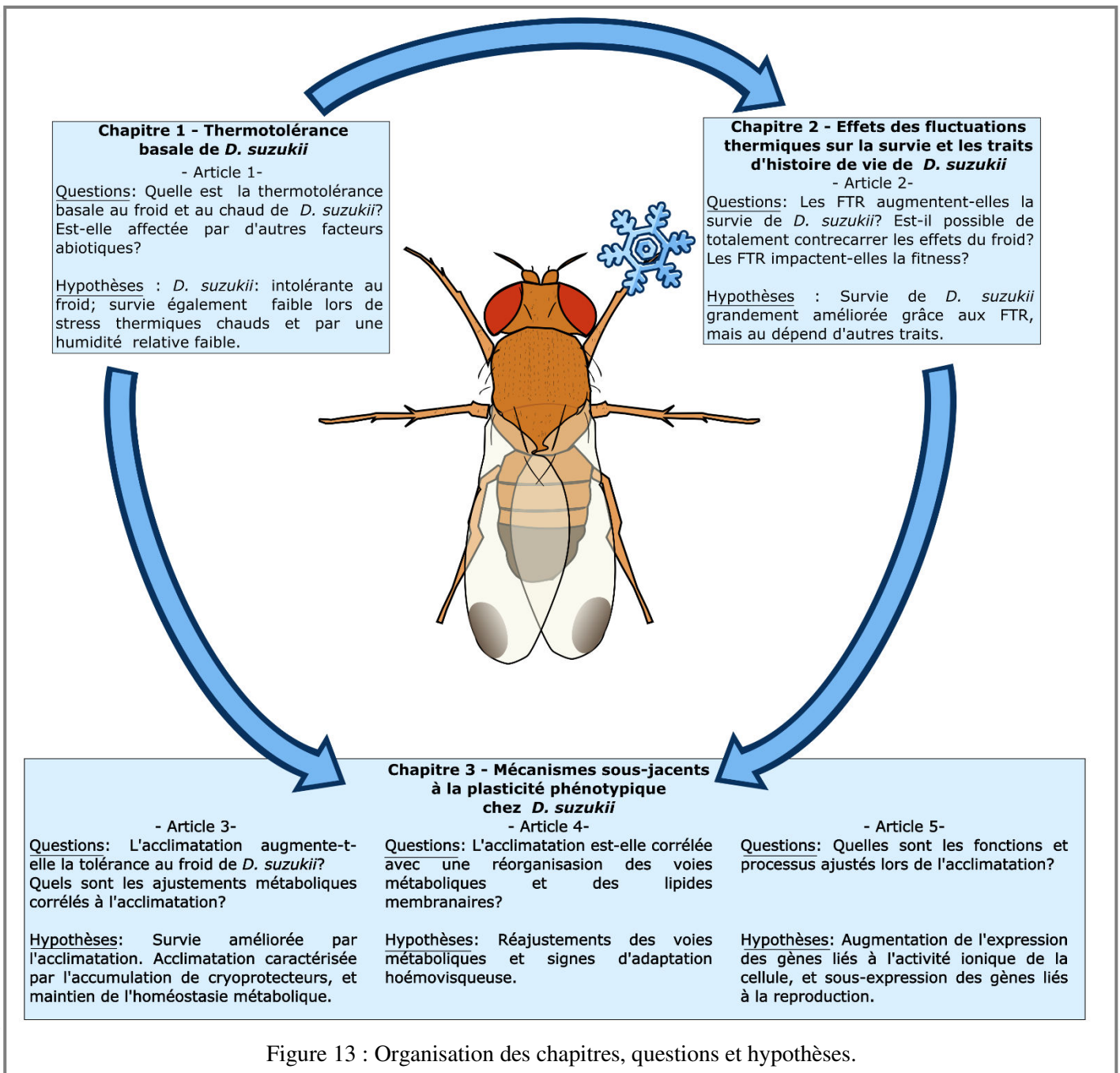
Par conséquent, les objectifs de ma thèse étaient :

- 1- Augmenter nos connaissances sur la thermotolérance basale (au froid et au chaud) de *Drosophila suzukii*.
- 2- Evaluer la plasticité de la tolérance au froid chez cette espèce, par l'utilisation de fluctuations thermiques et de différents processus d'acclimatation.
- 3- Identifier les mécanismes physiologiques sous-jacents à l'induction de la tolérance au froid (notamment par acclimatation) à l'aide de techniques « omiques » (transcriptomiques, lipidomiques, métabolomiques).

D'après nos connaissances de la biologie de *D. suzukii* et de la thermobiologie des insectes nos hypothèses étaient les suivantes :

- *Drosophila suzukii* est une espèce intolérante au froid, sa survie devrait être sévèrement impactée lors de stress thermiques froids et chauds.
- La tolérance au froid de *D. suzukii* est extrêmement plastique.
 - x Les fluctuations thermiques devraient pouvoir compenser les effets délétères des températures froides, mais pourraient en contrepartie altérer les traits d'histoire de vie et donc la valeur adaptative des individus.
 - x L'acclimatation et notamment l'acclimatation développementale devrait largement promouvoir la tolérance au froid de cette espèce.
- La plasticité de la thermotolérance de *D. suzukii* reposerait sur des mécanismes physiologiques communs aux ectothermes, tels que :
 - x L'adaptation homéovisqueuse des membranes cellulaires
 - x La synthèse de cryoprotecteurs
 - x Un réajustement des voies métaboliques tel que le cycle de Krebs ou la glycolyse
 - x Le maintien de l'homéostasie métabolique ionique et hydrique

L'organisation des différents chapitres de cette thèse, ainsi que les différentes questions et hypothèses qui y seront traitées sont disponibles dans la Figure 13.



Chapitre 1 : Thermotolérance basale de *D. suzukii*

Article 1: Basal tolerance to heat and cold exposure of the spotted wing drosophila, *Drosophila suzukii*

Contexte et objectif du chapitre :

La température constitue un élément majeur de la niche fondamentale des organismes (voir partie **I.2 de l'introduction**). Etudier les limites de la thermotolérance des espèces invasives est donc essentiel afin d'estimer l'évolution de leur aire de répartition. *D. suzukii* est une espèce invasive, intolérante au froid, pourtant elle est capable de survivre aux températures hivernales sous nos latitudes. Sa thermotolérance est donc encore mal comprise. De plus, la plupart des études portant sur ce sujet traitaient de la tolérance au froid de souches Nord-Américaines, la thermotolérance des souches Européennes restait donc peu connue. Concernant sa tolérance au chaud, les données disponibles étaient encore plus limitées. Par conséquent, l'objectif de ce chapitre était d'augmenter nos connaissances fondamentales sur la thermotolérance basale, au chaud et au froid de pupes et d'adultes *D. suzukii*.

Informations supplémentaires :

Les figures et tableaux supplémentaires mentionnés dans cet article sont disponibles en **annexe 2** et également accessibles en suivant ce lien :

<https://peerj.com/articles/3112/#supplemental-information>

Résumé de l'article en français :

La drosophile à ailes tachetées, *Drosophila suzukii*, est un nouveau ravageur de culture en Europe et Amérique du Nord qui cause d'importants dégâts, notamment aux productions de fruits rouges. La température et l'humidité sont parmi les paramètres abiotiques les plus importantes pour le développement et la valeur adaptative des insectes. Dans bien des situations, la température peut s'avérer stressantes pour les insectes et peut donc compromettre leur survie. La capacité d'un organisme à faire face à ces variations de températures stressantes dépend de sa thermotolérance basale. Des connaissances concernant les effets de la température sur la survie de *D. suzukii* sont requises pour pouvoir lutter efficacement contre ce ravageur. L'objectif de ces travaux étaient donc d'étudier la tolérance basale au froid et au chaud de *D. suzukii*. Des adultes et des pupes ont été soumis à six températures froides (allant de -5 à 7,5°C) et sept températures chaudes (de 30 à 37°C) en faisant varier les durées d'expositions, nous

permettant d'étudier la relation survie x durée d'exposition x température. Les mâles étaient globalement plus tolérants au froid que les femelles. Au-dessus de 5°C, la mortalité des adultes était négligeable, même lors d'expositions prolongées (seulement 20% de mortalité après un mois à 7,5°C). La tolérance au chaud des mâles était plus faible que celle des femelles aux températures les plus hautes (34, 35 et 37°C). Les pupes étaient bien moins tolérantes au froid que les adultes à toutes les températures testées (Lt₅₀ à 5°C : 4-5 jours pour les adultes vs 21 h pour les pupes). Toutefois, les pupes étaient beaucoup plus tolérantes au chaud que les adultes pour les températures les plus extrêmes (Lt₅₀ à 37°C : 30 min pour les adultes, vs 4 h pour les pupes). Par la suite, nous avons évalué la thermotolérance des pupes selon deux conditions d'humidité relative (faible ou haute). Une faible humidité relative n'impactait pas la tolérance au froid des pupes, mais réduisait considérablement leur tolérance au chaud. Ces résultats démontrent que la survie de *D. sukii* lors d'expositions au froid et au chaud dépend de l'intensité du stress, de la durée d'exposition, du genre et du stade de développement. L'approche expérimentale utilisée dans cette étude offre une description complète de la thermotolérance basale de *D. sukii*.

Basal tolerance to heat and cold exposure of the spotted wing drosophila, *Drosophila suzukii*

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ABSTRACT

The spotted wing Drosophila, *Drosophila suzukii*, is a new pest in Europe and America which causes severe damages, mostly to stone fruit crops. Temperature and humidity are among the most important abiotic factors governing insect development and fitness. In many situations, temperature can become stressful thus compromising survival. The ability to cope with thermal stress depends on basal level of thermal tolerance. Basic knowledge on temperature-dependent mortality of *D. suzukii* is essential to facilitate management of this pest. The objective of the present study was to investigate *D. suzukii* basal cold and heat tolerance. Adults and pupae were subjected to six low temperatures (−5–7.5 °C) and seven high temperatures (30–37 °C) for various durations, and survival-time-temperature relationships were investigated. Data showed that males were globally more cold tolerant than females. At temperature above 5 °C, adult cold mortality became minor even after prolonged exposures (e.g., only 20% mortality after one month at 7.5 °C). Heat tolerance of males was lower than that of females at the highest tested temperatures (34, 35 and 37 °C). Pupae appeared much less cold tolerant than adults at all temperatures (e.g., Lt₅₀ at 5° C: 4–5 d for adults vs. 21 h for pupae). Pupae were more heat tolerant than adults at the most extreme high temperatures (e.g., Lt₅₀ at 37 °C: 30 min for adults vs. 4 h for pupae). The pupal thermal tolerance was further investigated under low vs. high humidity. Low relative humidity did not affect pupal cold survival, but it reduced survival under heat stress. Overall, this study shows that survival of *D. suzukii* under heat and cold conditions can vary with stress intensity, duration, humidity, sex and stage, and the methodological approach used here, which was based on thermal tolerance landscapes, provides a comprehensive description of *D. suzukii* thermal tolerance and limits.

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Additional Information and
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INTRODUCTION

Temperature and water availability are among the most important factors influencing animal distribution, reproduction, and fitness (Chown & Nicolson, 2004; Angilletta Jr, 2009) and has therefore a great influence on the invasive success of alien species (Bellard et al., 2013). The spotted wing Drosophila (SWD), *Drosophila suzukii* (Matsumara), is a new insect pest in Western Europe and North America that causes severe damage to a large range of fruit plants, and especially stone fruit crops (Walsh et al., 2011; Asplen et al., 2015).

This invasive species is native to Southeast Asia and has been introduced in Spain, Italy, and North America in 2008 (Hauser, Gaimari & Damus, 2009; Raspi et al., 2011; Calabria et al., 2012). It is now widely distributed in West Europe (Cini, Ioratti & Anforta, 2012) and both in United States and in Southern Canada (Hauser, 2011). While most *Drosophila* species oviposit in rotting fruits, SWD females prefer to oviposit in ripe fruits (Kanzawa, 1939; Mitsui, Takahashi & Kimura, 2006). A sclerotized ovipositor allows flies to pierce through skin fruit (Hauser, Gaimari & Damus, 2009), and lay their eggs into a very large host range (Cini, Ioratti & Anforta, 2012; Poyet et al., 2015). The damages that larvae cause to fruits can have huge economic impact (Goodhue et al., 2011). Insect resistance to chemicals, frequent applications of insecticides owing to SWD short generation time and concerns about public health are considerable issues that have turned research towards non-chemical, environmentally friendly approaches. Sterile Insect Technique (SIT) or Incompatible Insect Technique (IIT) are innovative methods that offer sustainable opportunities to regulate pest populations (Zabalou et al., 2004; Dyck, Hendrichs & Robinson, 2005). Efforts are currently underway to develop such techniques in SWD (H Colinet, pers. comm., 2017). These technologies require release of a huge number of competitive insects produced at industrial scale. A successful application of SIT and/or IIT thus requires basic knowledge on thermal biology of SWD to develop cold storage methods and adapt prior-release mass-rearing protocols to the temperature within the release site(s) (*e.i.* in greenhouse).

It is assumed that the success of SWD invasion is partly due to a series of adaptations to temperate climates (Rota-Stabelli, Blaxter & Anfora, 2013). For instance, this species is freeze-intolerant and chill-susceptible (Kimura, 2004; Dalton et al., 2011; Jakobs, Garipey & Sinclair, 2015; Ryan et al., 2016; Plantamp et al., 2016) but has a large thermal tolerance plasticity which likely favors its overwintering (Jakobs, Garipey & Sinclair, 2015). Another hypothesis for explaining its overwintering success in cold regions is that adults may take refuge into human-made structures or migrate to suitable microclimates during cold periods (Kanzawa, 1939; Kimura, 2004; Cini, Ioratti & Anforta, 2012; Rota-Stabelli, Blaxter & Anfora, 2013; Zerulla et al., 2015). SWD overwinters as adult dark winter morph (Kanzawa, 1936; Stephens et al., 2015; Shearer et al., 2016; Toxopeus et al., 2016; Wallingford & Loeb, 2016). This morph is characterized by arrested reproduction and increased cold tolerance (Stephens et al., 2015; Toxopeus et al., 2016; Shearer et al., 2016; Wallingford & Loeb, 2016), but it is not yet clear whether this morph entails a true reproductive diapause or not (Toxopeus et al., 2016; Wallingford & Loeb, 2016; Zhai et al., 2016). Most recent studies on SWD cold tolerance were designed to understand overwintering strategy in newly infested cold regions, in order to better predict invasion potential or winter survival probability (*e.i.* Dalton et al., 2011; Stephens et al., 2015; Zerulla et al., 2015; Shearer et al., 2016; Wallingford & Loeb, 2016). In most of these studies, cold survival was assessed by subjecting insects either to a single low temperature with different durations (*e.i.* Jakobs, Garipey & Sinclair, 2015; Toxopeus et al., 2016), or to a series of low temperatures but with a single duration of exposure (*e.i.* Kimura, 2004; Ryan et al., 2016; Wallingford & Loeb, 2016). From a conceptual perspective, this can be questionable as the impact of any stress depends on its intensity and duration. Hence, investigating a single parameter may not be sufficient to describe a response that is embedded in two dimensions (Rezende, Castañeda

de Santos, 2014). To fully appreciate the innate capacity of a species to cope with cold and heat stress, an approach based on tolerance landscape was suggested by Rezende, Castañeda de Santos (2014). The thermal tolerance landscape (TTL), describes the probability to survive thermal stress as a function of both the intensity and the duration. In the present study, this approach was adopted to describe basal heat and cold tolerance of SWD at adult and pupal stage. Previous works have reported basic information on heat tolerance of SWD in native SWD populations (Kanzawa, 1939; Kimura, 2004). Kanzawa (1939) found a decrease of SWD motor activity when exposed to 30 °C, and Kimura (2004) exposed flies at different temperatures for a single duration of 24 h and estimated the 50% lethal temperature to be at 32 °C. Since invasion process may trigger adaptation to novel climatic areas, data are highly needed on invasive populations submitted to a range of thermal stress intensities and durations. In *Drosophila melanogaster*, the humidity during thermal stress modifies survival probability and the response can be temperature-dependent (Bubliy et al., 2012; Kobey & Montooth, 2013). Combining high temperature with low humidity provides more stressful conditions to *Drosophila* flies than high temperature with high humidity (Bubliy et al., 2012). Furthermore, increasing humidity during cold exposure increased survival at 6 °C, but not at −4 °C in *D. melanogaster* (Kobey & Montooth, 2013). This underlines that interaction among abiotic factors may be complex. In the present study, we also investigated the effect of different relative humidity levels (low vs. high) on cold and heat tolerance of SWD pupae. The general assumptions of this study were: (1) SWD survival would be a function of both temperature stress intensity and time exposure. (2) Because thermal performance curves are nonlinear and asymmetric (Colinet et al., 2015a), we predicted uneven effects of increasing intensity of cold and heat stress. More specifically, we predicted that survival would decrease rather progressively with cold stress intensity, and more abruptly with heat stress intensity. (3) We also expected differential responses between sexes and stages. Based on previous data from *D. melanogaster* (Jensen, Overgaard & Sørensen, 2007), we predicted that pupae would be less thermotolerant than adults. (4) Finally, we predicted that desiccating condition during thermal stress would further reduce survival probability compared to thermal stress under high humidity.

MATERIALS AND METHODS

Flies origin and rearing

The SWD strain is initially based on a mixed population from infested fruits collected from different locations in the south part of the Sugana Valley (Trentino, Italia), and brought to the Vigalzano station of the Edmund Mach foundation (46°04'25.74"N 11°13'52.45"E) in 2011. This strain was then exported in our laboratory (Rennes/France) in early 2016 where it has been continuously mass-reared in high numbers. For experimentations, SWD was reared in glass bottle (100 mL) and supplied with standard diet (for 1 l: agar: 15 g, sucrose: 50 g, brewer yeast: 40 g, cornmeal: 40 g, kalmus: 8 g, Nipagin: 8 mL). At least 12 bottles (each containing 100–300 flies) were used to maintain the strain, and flies from different bottles were crossed every generation to limit inbreeding. Bottles were kept in incubators (Model MIR-154-PE; PANASONIC Healthcare Co., Ltd. Gunma, Japan) at 25

°C, 65–70% RH, 12L : 12D. Adults and pupae randomly taken from the rearing stock were used in experiments. In the present study, we focused on adults and pupae as these two stages can be isolated from the food and are thus more convenient for cold storage than larvae. All tested adults were between 5- and 7-days-old to avoid age-related differences in stress tolerance (Colinet *et al.*, 2015b). Males were separated from females visually (with an aspirator) without CO₂ to avoid stress due to anesthesia (Colinet & Renault, 2012). For pupae, individuals that had pupated for a maximum of 48 h were used (i.e., corresponding to eight days after egg laying at 25 °C).

Thermal tolerance assays

Flies and pupae were subjected to six low constant temperatures (−5, −2.5, 0, 2.5, 5 and 7.5 °C) and seven high constant temperatures (30, 31, 32, 33, 34, 35 and 37 °C) for various durations. At least seven durations were used for each temperature and these are provided in Tables S1–S5 for each experiment. Exposure durations were pre-determined with preliminary assays, in order to obtain for each temperature a range of survival spanning from 0% for the shortest exposure to 100% for the longest exposure. At the most stressful temperatures (at heat and cold), additional time points were considered because mortality occurred very quickly (within less than 2 h) (see Tables S1–S5). For each sampling duration, three replicates of 10 flies or 10 pupae, randomly taken from the rearing stock, were used. Flies and pupae were exposed to the different thermal conditions either using food vials placed in incubators (Model MIR-154-PE; PANASONIC Healthcare Co., Ltd. Gunma, Japan) for the longer assays (2.5, 5, 7.5, 30, 31, 32 °C) or using glass vials immersed in a glycol solution cooled by a cryostat (Cryostat Lauda ECO RE 630) for the shorter assays (−5, −2.5, 0, 33, 34, 35, 36, 37 °C). Males and females were exposed in separated vials. Temperature was checked during all assays using thermocouple K (measuring accuracy: ±0.5 °C) connected Testo thermometers (Model 175 T3; TESTO Limited, Hampshire, England) placed into the same experimental vial without flies. After stress exposure, SWD adults were allowed to recover in 40 mL food vials under standard rearing conditions (25 °C, 65–70% RH, 12L : 12D). Adult survival was assessed by counting the proportion of dead and living individuals 24 h post exposure. For pupae, the results were expressed as a percentage of eclosion, considered here as a proxy of survival. Flies were considered as alive when the adult totally emerged from the puparium. Because isolation and manipulation of pupae in the preparation of thermal assays might cause some damage to the puparium, five sets of 20 untreated pupae were kept at 25 °C to estimate possible mortality due to manipulation.

Thermal stress under high and low relative humidity

In this experiment, we used only pupae to assess the impact of humidity (low vs. high) during thermal stress. Indeed, there was a technical limitation with the application of low RH in adults, as it was impossible to generate low RH (5–10%) with presence of food within the vials. Pupae were exposed to four different low constant temperatures (0, 2.5, 5 and 7.5 °C) and five different high constant temperatures (32, 33, 34, 35 and 37 °C) either under a high (80–100%) or low (5–10%) relative humidity (RH). For each sampling duration, a set

of 15 pupae randomly taken from the rearing stock was used. To produce high RH, a cotton ball saturated with water was placed at the bottom of a 50 mL closed centrifugation tube. For low RH, dehydrated silicagel was placed at the bottom of a 50 mL tube. Foam slices were added to the devices to prevent direct contact of SWD pupae with cotton or silicagel. RH and temperatures were checked directly into experimental tubes using Ibutton's Hygrochron (Maxim Integrated, San Jose, CA, USA), and K thermocouples (measuring accuracy: ± 0.5 °C) connected to Testo thermometers (Model 175 T3; TESTO Limited, Hampshire, England). As previously described, preliminary assays were first performed in order to calibrate the number and duration of sampling times to get emergence data spanning from 0 to 100%. Again, at least seven durations were used for each tested temperature (details in [Table S5](#)). Flies were considered as alive when the adult eclosed from the puparium.

Statistical analyses

Survival data were modeled in R (*R Core Team, 2016*) by specifying a generalized linear model (GLM) with logistic link function for proportions outcome (i.e., number of dead/alive per vial). The response variable was dependent on stress duration, temperature, sex (for adults), RH treatment (when tested), and all the interactions. Full factorial models were used, and the effects of each variable were analyzed through an Analysis of Deviance ("Anova" function in "car" package, (*Fox & Weisberg, 2011*)). The 50% median lethal times (Lt_{50}) for each temperature were calculated as follow:

$$Lt_{50} = \frac{\text{logit}(0.5) - a}{b} \quad \text{Venables \& Ripley (2002).}$$

where a and b respectively correspond to the intercept and the slope of each condition GLM's prediction. 95% confidence intervals around estimated Lt_{50} were retrieved by resampling model parameters (10,000 iterations, "arm" package, (*Gelman & Su, 2014*)). Lt_{50} values represent the time at which 50% of the tested individuals are dead at a given temperatures. Even if is a standard and useful proxy to describe and compare thermal tolerance data, it is important to consider the entire range of probabilities and not only 50% survival. Therefore, to complement this information, the predicted values acquired from GLMs as function of both stress intensity and duration were also represented using 3D plots, following the thermal tolerance landscape (TTL) approach suggested by *Rezende, Castañeda & Santos (2014)*. All the 3D representations of time \times temperature \times survival are available in [Figs. S1](#) and [S2](#). Finally, to help interpreting all the terms of the GLMs, effect plot function in the package "effects" (*Fox, 2003*) was used. These effect plots show the conditional coefficients ("marginal effects") for all variables and interaction terms. All the effect plots are available in [Figs. S3–S8](#) for each experiment separately.

RESULTS

SWD cold tolerance

Control mortality of untreated pupae didn't exceed 1%. In both adults and pupae, 100% mortality was reached for all tested temperatures, except for adults kept at 7.5 °C. The multiple panels [Fig. 1](#) illustrates cold survival data in adults (males and females, [Fig. 1A](#))

and in pupae (Fig. 1B) according to the different tested temperatures and durations. Temperature and duration had strong effects on adult cold survival ($\chi^2 = 856.36$, $df = 5$, $p < 0.001$; $\chi^2 = 502.59$, $df = 1$, $p < 0.001$, respectively). Survival decreased with decreasing temperature and with increasing exposure duration (Fig. 1A, Figs. S1 and S3). Furthermore, at the lowest temperatures, temporal reduction of survival was greatly reduced (duration \times temperature interaction; $\chi^2 = 1075.71$, $df = 5$, $p < 0.001$; Figs. S1 and S3). Males were globally more cold-tolerant than females ($\chi^2 = 99.95$, $df = 1$, $p < 0.001$; Figs. S1 and S3). Sexes were differentially affected by decreasing temperatures (temperature \times sex interaction; $\chi^2 = 41.63$, $df = 5$, $p < 0.001$), with females more affected than males at the lowest tested temperatures (Fig. 1A, Figs. S1 and S3). Temporal changes of survival were similar between sexes (no sex \times duration interaction; $\chi^2 = 0.65$, $df = 1$, $p > 0.05$).

Temperature and duration also had a strong impact on pupal cold survival ($\chi^2 = 156.68$, $df = 5$, $p < 0.001$; $\chi^2 = 463.94$, $df = 1$, $p < 0.001$, respectively) (Fig. 1B). Survival significantly decreased with decreasing temperature and with increasing exposure duration (Fig. 1B, Figs. S1 and S4). The temporal decrease in survival was dependent on temperature (duration \times temperature interaction; $\chi^2 = 161.43$, $df = 5$, $p < 0.001$), it was much faster at lower temperatures (Fig. 1B, Figs. S1 and S4).

Lt₅₀ for adults and pupae at the different tested low temperatures are provided in Fig. 2. Based on Lt₅₀ values and their confidence intervals, pupae appeared much less cold-tolerant than adults (Fig. 2). For adults, models weren't able to estimate Lt₅₀ at 7.5 °C, as 80% of flies survived the 30 days of cold exposure (i.e., 50% mortality was not reached) (see Fig. 1A).

SWD heat tolerance

For both adults and pupae, 100% mortality was reached for all tested temperatures, except for pupae at 30 and 31 °C, where five and four individuals, respectively, eclosed during the last day of the experiment and were then considered as alive. The multiple panels Fig. 3 illustrates survival data in adults (males and females, Fig. 3A) and in pupae (Fig. 3B) according to the different tested temperatures and durations. Temperature and duration had strong effect on adult heat survival ($\chi^2 = 819.69$, $df = 6$, $p < 0.001$; $\chi^2 = 889.77$, $df = 1$, $p < 0.001$, respectively). Survival decreased with increasing temperature and increasing exposure duration (Fig. 3A and Fig. S5). Furthermore, at highest temperatures, temporal reduction of survival was much faster than at milder temperatures (duration \times temperature interaction; $\chi^2 = 1495.20$, $df = 6$, $p < 0.001$; Figs. S1 and S5). There was no difference between males and females ($\chi^2 = 1.52$, $df = 1$, $p > 0.05$). Yet sexes were differentially affected by increasing temperature (temperature \times sex interaction; $\chi^2 = 94.43$, $df = 6$, $p < 0.001$), with males more affected than females at the highest temperatures (Fig. 3A, Figs. S1 and S5), indeed at 34, 35 and 37 °C, mid-time survival of female was at least twice greater than that of males (Fig. 3A). Temporal decreases of survival were similar between sexes (no sex \times duration interaction, $\chi^2 = 0.19$, $df = 1$, $p > 0.05$).

Temperature and duration also had a strong impact on pupal heat survival ($\chi^2 = 210.72$, $df = 6$, $p < 0.001$; $\chi^2 = 388.71$, $df = 1$, $p < 0.001$, respectively) (Fig. 3B). Survival significantly decreased with increasing temperature and with increasing exposure duration (Fig. 3B, Figs. S1 and S6). The temporal decrease in survival was dependent on temperature

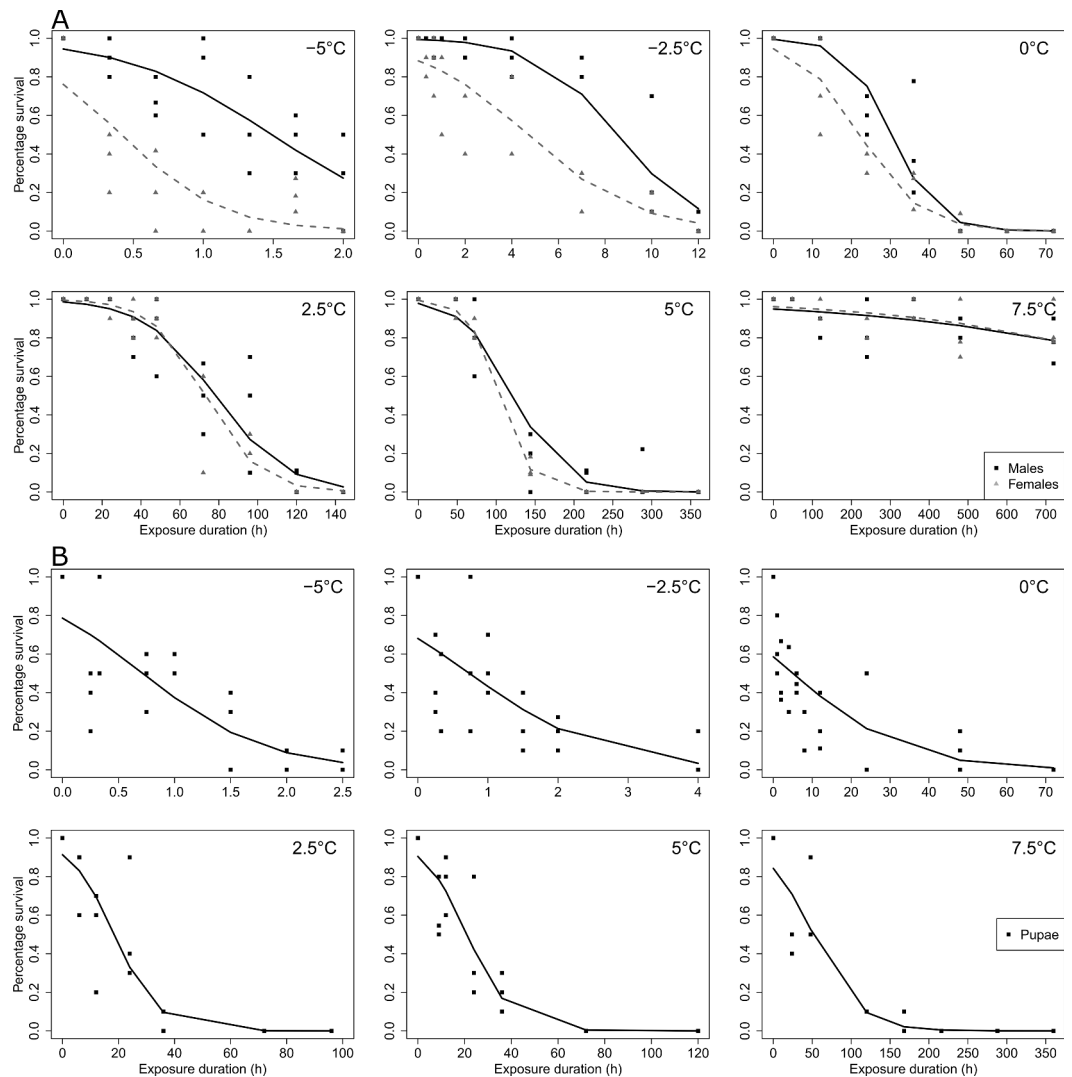


Figure 1 SWD survival as a function of low temperature and duration exposure. (A) adults; (B) pupae. Points correspond to observed data, and lines to GLM predictions. In (A), black solid lines correspond to male's predictions, and grey dashed lines to females predictions. In (B) black solid lines correspond to pupae's predictions. The tested temperatures are indicated in the left top corner of each plot.

(duration \times temperature interaction; $\chi^2 = 662.25$, $df = 6$, $p < 0.001$), it was also much faster at higher tested temperatures. This latter effect is illustrated by the duration of tests which clearly reduced as the tested temperature increased (e.g., max 100 h at 32 °C vs. max 10 h at 37 °C) (Fig. 3B).

Lt_{50} for adults and pupae at the different high temperatures are provided in Fig. 4. Based on Lt_{50} values and their confidence intervals, pupae appeared much less tolerant than adults at temperatures under 33 °C; however, they were more tolerant than adults at temperatures above 33 °C (Fig. 4).

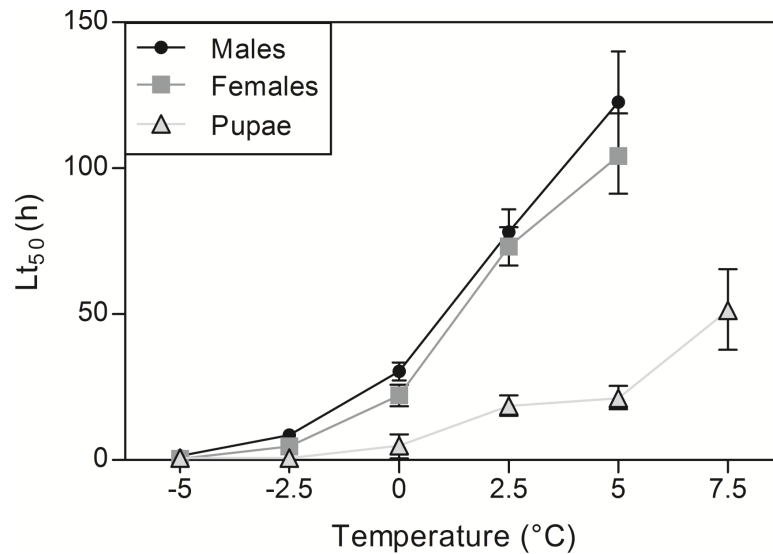


Figure 2 Estimated Lt_{50} values for each tested low temperature. Males, females and pupal Lt_{50} values \pm 95% confidence intervals. Lt_{50} is the time at which 50% of the population is dead. Lt_{50} values are calculated from GLMs.

Interaction between relative humidity and thermal stress

Low and high temperature treatments were statistically analyzed separately. Under cold exposure, 100% mortality was reached for all tested temperatures for both high and low RH. The multiple panels Fig. 5 illustrates survival data in pupae according to the different tested temperatures (A: cold; B: heat) and durations. Temperature and duration had strong effect on pupal cold survival ($\chi^2 = 91.74$, $df = 3$, $p < 0.001$; $\chi^2 = 649.88$, $df = 1$, $p < 0.001$, respectively). Cold survival decreased with decreasing temperature and with increasing exposure duration (Fig. 5A, Figs. S2 and S7). Furthermore, at the lowest temperatures, temporal reduction of survival was much faster (duration \times temperature interaction; $\chi^2 = 68.07$, $df = 3$, $p < 0.001$; Figs. S2 and S7). RH did not differentially affect cold survival ($\chi^2 = 0.02$, $df = 1$, $p > 0.05$), but temporal changes were different between RH levels: survival decrease was slightly faster in dry than in humid condition (RH \times duration interaction; $\chi^2 = 5.48$, $df = 1$, $p < 0.05$; Figs. S2 and S7).

Under heat exposure, 100% mortality was also reached for all tested temperatures for both high and low RH (Fig. 5B). Temperature and duration had again strong effects on pupae heat survival ($\chi^2 = 306.20$, $df = 4$, $p < 0.001$; $\chi^2 = 831.90$, $df = 1$, $p < 0.001$, respectively). Survival decreased with increasing temperature and with increasing exposure duration (Fig. 5B, Figs. S2 and S8). Furthermore, at the highest temperatures, temporal reduction of survival was much faster (duration \times temperature interaction; $\chi^2 = 83.46$, $df = 4$, $p < 0.001$). RH greatly affected heat survival ($\chi^2 = 95.97$, $df = 1$, $p < 0.001$), with survival being significantly higher when pupae were exposed to high vs. low RH (Fig. 5B, Figs. S2 and S8). In addition, RH interacted with both temperature and duration ($\chi^2 = 19.25$, $df = 4$, $p < 0.001$; $\chi^2 = 14.17$, $df = 1$, $p < 0.001$, respectively). Survival decreased with increasing temperature and this thermal-dependent process was more

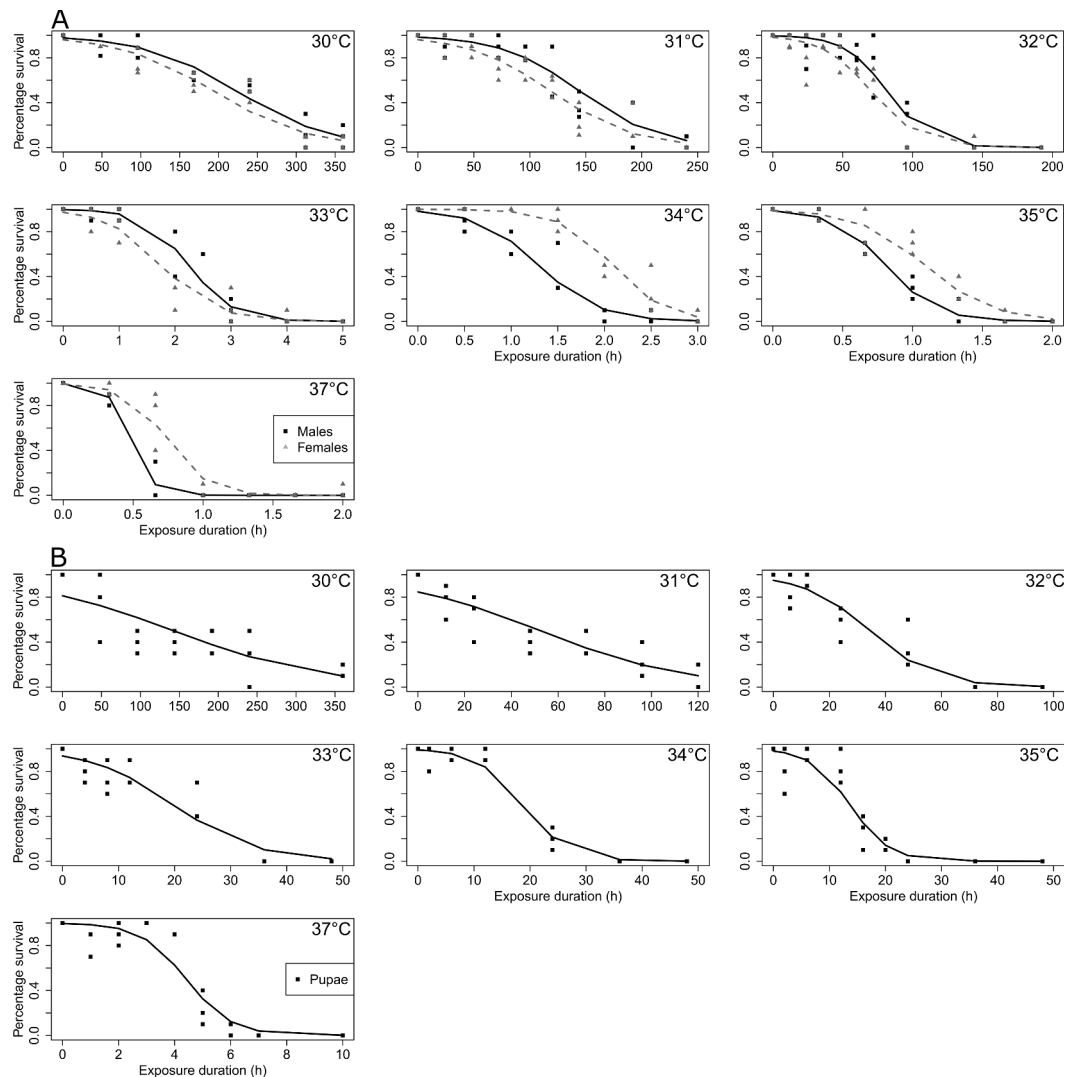


Figure 3 SWD survival as a function of high temperature and duration exposure. (A) adults; (B) pupae. Points correspond to observed data, and lines to GLM predictions. In (A), black solid lines correspond to male's predictions, and grey dashed lines to female's predictions. In (B), black solid lines correspond to pupae's predictions. The tested temperatures are indicated in the left top corner of each plot.

severe under low RH (Fig. 5B, Figs. S2 and S8). In addition, temporal decrease in survival (across all temperatures) was globally faster under low RH. Based on Lt_{50} values and their confidence intervals, it appeared that low RH greatly diminished heat survival, but did not affect cold survival (Fig. 6).

DISCUSSION

In the present study, the basal thermal tolerance of SWD was studied considering adult and pupal survival as a function of both stress intensity (heat and cold) and exposure duration. A reduction in survival with increasing thermal stress intensity and duration was observed, both under low and high temperatures. This decrease is consistent with the

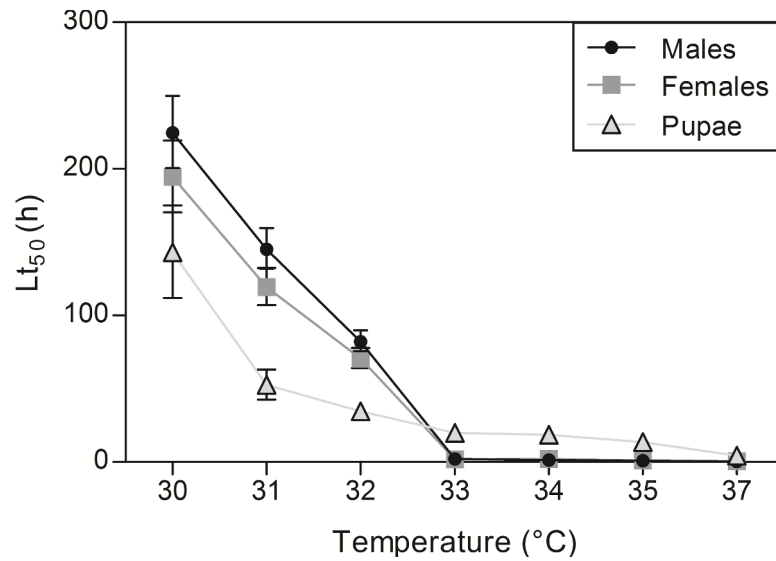


Figure 4 Estimated Lt_{50} values for each tested high temperature. Males, females and pupal Lt_{50} values \pm 95% confidence intervals. Lt_{50} is the time at which 50% of the population is dead. Lt_{50} values are calculated from GLMs.

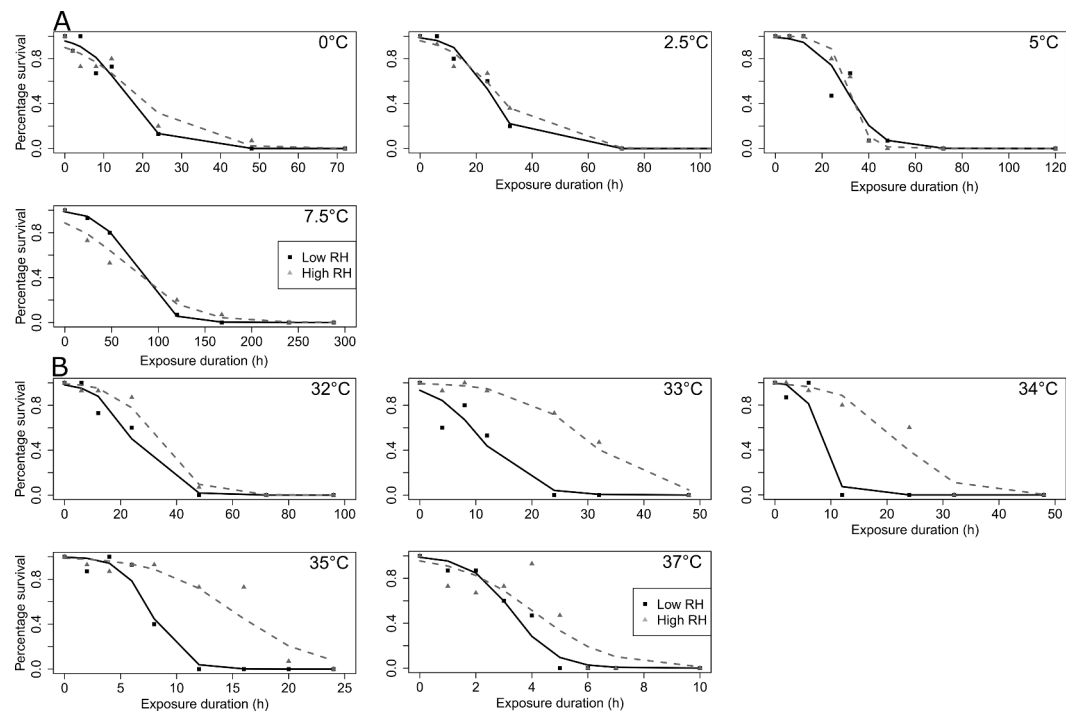


Figure 5 SWD pupal survival as a function of temperature and exposure duration under two relative humidity (RH) levels. (A) Cold exposure; (B) Heat exposure. Points correspond to observed data, and lines to GLM predictions. The tested temperatures are indicated in the left top corner of each plot. Black solid lines: low RH, grey dashed lines: high RH.

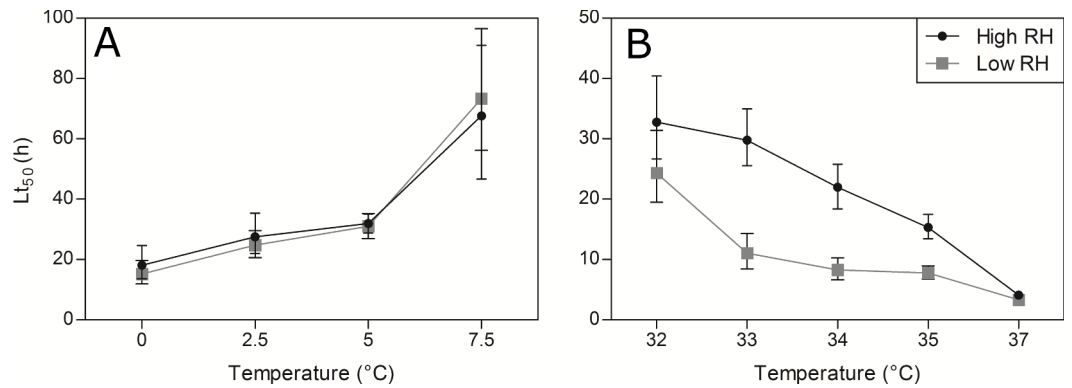


Figure 6 Pupal Lt_{50} values at low and high temperatures under two relative humidity (RH) levels. Lt_{50} values \pm 95% confidence interval values for low (A) and high temperatures (B). Lt_{50} values are calculated from previous GLM predictions.

classical dose–response relationship where survival declines with the “dose of stress” which is considered here as a combination of temperature stress intensity and duration (Colinet, Lalouette & Renault, 2011; Rezende, Castañeda & Santos, 2014).

The cold tolerance of SWD estimated in the present study was rather consistent with previously reported data. Indeed, it appeared that adult and pupal mortality occurred very rapidly at subzero temperatures (see Figs. 1 and 2). This fits with early reports that also found very short survival durations at subzero temperatures (*e.i.* Jakobs, Garipey & Sinclair, 2015; Stephens *et al.*, 2015; Plantamp *et al.*, 2016; Ryan *et al.*, 2016). These data corroborate that SWD is a chill susceptible species that does not tolerate brief exposures to sub-zero temperatures (Kimura, 2004; Dalton *et al.*, 2011; Jakobs, Garipey & Sinclair, 2015; Ryan *et al.*, 2016; Plantamp *et al.*, 2016). At 0 °C, it required about one day to reach 50% mortality in adults and much less time was needed (approximately 5 h) in pupae. This is also in the range of previous data on SWD (Kimura, 2004; Jakobs, Garipey & Sinclair, 2015; Plantamp *et al.*, 2016); however, the values described in this study appear slightly lower than those reported in other studies and strains. These slight variations in thermotolerance may be related to different rearing conditions. Indeed, we reared flies at 25 °C not at 21 °C as in other laboratories (Jakobs, Garipey & Sinclair, 2015; Plantamp *et al.*, 2016). Variations may also result from different local adaptations of the tested strains (Hoffmann, Anderson & Hallas, 2002; Van Heerwaarden *et al.*, 2012). Finally, our strain was reared under laboratory conditions since 2011, and hence, we cannot exclude an effect of inbreeding on stress tolerance, despite constant efforts to mitigate this issue. At temperatures above 0 °C, several days were required before reaching Lt_{50} . Interestingly, there appeared to be a sort of threshold between 5 and 7.5 °C where individuals shifted from detrimental condition (at 5 °C) to non-injurious condition (at 7.5 °C). Indeed, at 5 °C, Lt_{50} was reached in only five days, while at 7.5 °C, mortality remained low (under 20%) even after rather long exposure (one month). Previous data reported that non-acclimated SWD adults start to fall into coma at temperatures just below 5 °C (Jakobs, 2014). Therefore, it is likely that this temperature represents a physiological limit under which chilling injuries, such as neuromuscular dysfunctions, start to accumulate (Hazell & Bale, 2011; MacMillan

et al., 2012). In temperate regions, cold snaps with freezing events could be lethal to SWD. However, it seems that SWD overwinters as adults by migrating into protected microclimates, in leaf litter or in human made structures (Kanzawa, 1939; Kimura, 2004; Dalton *et al.*, 2011; Zerulla *et al.*, 2015; Rossi-Stacconi *et al.*, 2016). This avoiding strategy likely allows SWD to escape low winter temperatures *in natura*, and colonize new cool regions (Rota-Stabelli, Blaxter & Anfora, 2013; Asplen *et al.*, 2015).

Because thermal performance curves are nonlinear and asymmetric (skewed towards low temperatures) (Martin & Huey, 2008; Colinet *et al.*, 2015a), we predicted uneven effects of increasing the intensity of cold *vs.* heat stress. Essentially, we assumed that SWD survival will decrease rather progressively with increasing cold stress intensity, and we expected a steep decline in survival over certain limits under heat stress. Observation of Lt_{50} (Figs. 2 and 4) values and TTLs patterns (Fig. S1) support this assumption. Under low temperature conditions, a progressive survival decline was observed (Fig. 2 and Fig. S1), while under high temperature stress, there was clearly a limit over which survival crashed suddenly and became close to zero (Fig. 4 and Fig. S1). Indeed, at 32 °C, adult flies could sustain continuous heat stress for several days (Lt_{50} of 3–4 days), whereas at 33 °C, most flies succumbed within a couple of hours (Lt_{50} of about 2 h). Therefore 32 °C seem to be very close to critical thermal maximum for survival of SWD. Characteristically, the drop in performance (i.e., survival in this case) is generally more precipitous at supra-optimal temperatures than at sub-optimal temperatures (Denlinger & Yocum, 1998). Arrhenius-like effects can explain progressive and reversible changes of performance at sub-optimal temperatures, while the sharp decline at supra-optimal temperatures is generally ascribed to the destabilizing effects of heat on molecular interactions such as irreversible protein denaturation (Schulte, Healy & Fangué, 2011). These results are in accordance with early studies performed on different SWD populations. Kanzawa (1939) noticed a motor activity decrease of SWD when exposed to 30 °C, and Kimura (2004) estimated that the 50% lethal temperature at heat was around 32 °C. Also, the upper thermal limit for development is estimated to be at 31.5 °C (Asplen *et al.*, 2015). Ryan *et al.* (2016) found no adult hatching when Canadian flies developed at 31 °C. Therefore, our survival data together with the previous literature suggest that SWD is not a particularly heat-tolerant species. This likely explains the very low field survival of SWD when temperatures exceed 30 °C (Dalton *et al.*, 2011; Tochen *et al.*, 2014). While overwintering strategy is rather well studied (Kanzawa, 1936; Stephens *et al.*, 2015; Shearer *et al.*, 2016; Toxopeus *et al.*, 2016; Wallingford & Loeb, 2016), how flies cope with heat stress in the fields and more generally how SWD flies manage to survive under summer conditions (i.e., heat coupled with desiccation) is currently unknown.

Differences in thermotolerance between sexes and stages were expected. Under low temperature, SWD males were slightly more cold-tolerant than females. This contrasts with previous SWD studies which reported that females were more cold-tolerant than males (Kimura, 2004; Dalton *et al.*, 2011; Jakobs, Gariépy & Sinclair, 2015). However, lack of difference in cold tolerance between sexes were also reported in SWD (Ryan *et al.*, 2016). In *D. melanogaster*, the sex effect on cold tolerance can be sometimes in favor of males (Kelty & Lee, 2001; Sejerkilde, Sørensen & Loeschcke, 2003; Jensen, Overgaard & Sørensen,

2007), or females (David *et al.*, 1998; Condon *et al.*, 2015). These discrepancies may result from various factors, such as different measures of cold tolerance, tested temperatures or age of flies (Jensen, Overgaard & Sørensen, 2007). In consequence, our results suggest that in SWD, as in *D. melanogaster*, sexual dimorphism in various metrics of cold tolerance appears more as an idiosyncratic than a general rule (Gibert & Huey, 2001). This view is also supported by our observations that, under high temperature, we detected an interaction (sex \times temperature) that suggested that females better tolerated heat exposure but at certain temperatures (i.e., at the greatest temperatures).

Based on previous data from *D. melanogaster* (Jensen, Overgaard & Sørensen, 2007), we predicted that pupae would be less cold-tolerant than adults. Indeed, it appeared that pupal Lt_{50} values under cold conditions were consistently much lower than values of adults at all tested temperatures; and this was clearly visible on the shape of TTLs (Fig. S1). Furthermore, exposure to 7.5 °C induced high pupal mortality while it hardly affected adults. Other studies suggest that SWD pupae are less cold-tolerant than adults. For instance, Dalton *et al.* (2011) reported that pupae died earlier than adults when exposed to temperatures below 10 °C, and Ryan *et al.* (2016) found that SWD pupae could not survive 42 d of cold exposure contrary to adults. This seems coherent with the observation that in SWD, the overwintering stage is the adult in reproductive diapause (Stephens *et al.*, 2015; Zerulla *et al.*, 2015; Shearer *et al.*, 2016; Toxopeus *et al.*, 2016; Rossi-Stacconi *et al.*, 2016; Wallingford & Loeb, 2016). Ontogenetic differences under high temperature revealed different patterns than under low temperature. Pupae appeared less tolerant than adults to prolonged mild heat stress (temperatures < 33 °C), while under severe heat stress conditions (temperatures > 33 °C), pupae could sustain heat stress for much longer than adults. Indeed, under acute heat stress, pupae managed to survive exposures for several hours (from 10 to 20 h depending on the temperature), while adults succumbed in less than 2 h. In *Drosophila buzzatii*, pupae seem to be the most heat resistant stage, surviving temperatures above those that would kill all the other life stages (Krebs & Loeschcke, 1995). The high heat tolerance of pupae might be explained by Bogert effect (Huey, Hertz & Sinervo, 2003). According to this principle, behavioral thermoregulation allows ectothermic animals to escape lethal temperatures, thus less mobile stages (like pupae) have to cope with and tolerate more extremes conditions than mobile stages (Marais & Chown, 2008; Mitchell, Sinclair & Terblanche, 2013).

Finally, we predicted that contrasted RH levels will affect thermal tolerance patterns. Specifically, we expected that highly desiccating conditions during thermal stress will further reduce survival compared to stress conditions under high RH. As predicted, RH had strong impact on pupal survival, but this manifested only under heat stress. The Lt_{50} values illustrate these marked differences (Figs. 6A and 6B). Under cold conditions, the shapes of TTLs were globally identical between low and high RH, whereas under heat conditions, the TTLs were extended towards longer survival under high RH (Fig. S2). In *D. melanogaster*, the humidity during both heat (Bubliy *et al.*, 2012) and cold exposure (Kobey & Montooth, 2013) alters survival rate. Combining two stressors like high temperature with low RH provides more stressful conditions to vinegar flies than high temperature with high RH (Bubliy *et al.*, 2012). Here, the same synergetic-like phenomena was observed.

Prince & Parsons (1977) showed that under low RH, *D. melanogaster* adults move towards lower temperatures likely to mitigate water loss. It is reasonable to speculate that in natural conditions, SWD adults also tend to avoid heat stress and low RH by searching for protected and favorable microhabitats, but this remains to be tested. Recent mark-capture researches indicate that SWD achieves short-distance migrations from field margins to cultivated crops (*Klick et al., 2016*); therefore, migration towards favorable microclimates is completely conceivable. During the pupal stage, however, flies are immobile and are thus potentially subjected to prolonged heat and desiccation stress, particularly if pupation occurs outside of infested fruits, as is the case with SWD (*Asplen et al., 2015*). Despite being protected within the puparium, water loss by pupae can strongly affect survival of drosophilids, and even moderately dry conditions can induce substantial pupal mortality at permissive temperature (*Kojima & Kimura, 2003*). A recent study from *Tochen et al. (2016)* indicates that low RH (e.g., 20%) induced poor survival and lack of reproduction in SWD, suggesting that this species is particularly sensitive to water loss. Under low temperature, there was globally no effect of RH on pupal cold survival. Death during prolonged cold exposure may be due to a combination of stressors: low temperature, starvation and desiccation. If pupae were suffering from desiccation at cold, then altering RH during low temperature exposure should affect water loss, and therefore, the survival duration at cold. Lack of RH effect at cold suggests that desiccation is not a primary cause of mortality under cold stress.

In this work, basal thermal tolerance of SWD was studied considering survival as a function of temperature stress intensity (under heat and cold) and exposure duration in adults (males and females) and in pupae. It appeared that survival under heat and cold conditions was dependent on both stress intensity and duration, and hence, this study provides a comprehensive description and visualization of SWD thermal tolerance and limits. These results confirmed that SWD is a chill susceptible species. We found that at temperatures over 5 °C, adults managed to survive for rather long periods (one month). Tolerance to thermal stress over a range of conditions showed rather different perspectives: a sudden vs. a more progressive survival decline under heat vs. cold conditions, respectively. In particular, 32 °C seemed to be very close to critical thermal maximum for survival of SWD. A sexual dimorphism in thermal tolerance was also found but was temperature-dependent. Difference in thermal tolerance were also observed between stages, with pupae being drastically more sensitive to cold stress but more resistant to extreme heat stress than adults. Finally, data suggested that level of RH had strong impact on pupal survival under heat stress but not under cold stress.

Recently, a consortium of scientists has published a useful review with the updated situation of SWD all over the world (*Asplen et al., 2015*). The authors suggested a few directions for future research to improve the accuracy of SWD management. Acquisition of novel data on the biology of SWD at low temperature was highlighted as a priority, and we believe the present dataset may provide valuable elements in this regard. The present study is one of the first to provide a global description of SWD basal thermal tolerance, especially bringing new information about heat stress tolerance and the interaction between temperature and relative humidity. In a context of global climate change and considering

the winter temperature warming on temperate region, these results together with previous data from the literature highlight that SWD will most likely be able to tolerate mild cold stress conditions for several consecutive days (e.g., one month to 7.5 °C). This capacity may contribute to its overwintering success in novel invaded temperate regions. However, we wish to draw attention to the fact that stress tolerance data acquired from field-collected populations may deeply contrast with those resulting from laboratory-adapted lines (e.i. Hoffmann et al., 2001; Schou, Loeschcke & Kristensen, 2015). In consequence, despite logistical constraints, the next needed step is the realization of thermal studies on field-collected individuals.

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ADDITIONAL INFORMATION AND DECLARATIONS

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Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Thomas Enriquez conceived and designed the experiments, performed the experiments, analyzed the data, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Hervé Colinet conceived and designed the experiments, contributed reagents/materials/analysis tools, wrote the paper, reviewed drafts of the paper.

Data Availability

The following information was supplied regarding data availability:

Data: <https://figshare.com/s/e94eacc9d64dbbd47941>.

R scripts: <https://figshare.com/s/dba3a9c7eecb1d4603f3>.

Supplemental Information

Supplemental information for this article can be found online at <http://dx.doi.org/10.7717/peerj.3112#supplemental-information>.

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Chapitre 2 : Effet des fluctuations thermiques sur la survie et les traits d'histoire de vie de *Drosophila suzukii*

Article 2: Effects of fluctuating thermal regimes on cold survival and life history traits of the spotted wing *Drosophila* (*Drosophila suzukii*)

Contexte et objectif du chapitre :

Les résultats du **chapitre 1** ont montrés que des expositions prolongées au froid compromettent la survie de *D. suzukii*. Toutefois ces expositions ont eu lieu en températures constantes. Un grand nombre d'études ont montré que lorsque la période d'exposition était interrompue par des intervalles (des « pics ») de température chaude, la réponse des organismes était plus complexe, et conduisait à une diminution de la mortalité (cette technique est communément appelée régimes de température fluctuante – RTF, voir partie **I.7.d de l'introduction**). Même si ces procédés sont artificiels, ils peuvent mettre en évidence des réponses similaires à celles produites par les variations thermiques *in natura*. L'étude des RTF permet ainsi une approche des réponses mises en place par les insectes lors des variations de températures de leur environnement. Les RTF constituent également une bonne opportunité pour le stockage d'insectes à basse température, et s'avèrent ainsi grandement utiles en lutte biologique. Toutefois les RTF pourraient avoir un coût énergétique pour l'organisme, induisant des compromis affectant la qualité des insectes. Par conséquent, notre objectif lors de ce chapitre était d'évaluer l'impact des RTF sur la survie et les traits d'histoire de vie de *Drosophila suzukii*.

Informations supplémentaires :

Les figures et tableaux supplémentaires mentionnés dans cet article sont disponibles en **annexe 3** et sont également accessibles en suivant ce lien :

<https://figshare.com/s/ce881e4ace5524388b0f>

Résumé de l'article en français :

Drosophila suzukii est une drosophile invasive, causant d'importants dégâts à une large gamme de fruits cultivés. Afin de faciliter le biocontrôle de *D. suzukii* à l'aide de stratégies telles que la technique de l'insecte stérile ou de l'insecte incompatible, une production de masse de cette drosophile doit être mise en place, impliquant un stockage et des transports à basse température. Les dégâts provoqués par les températures froides peuvent être atténués par les RTF. Dans cette étude nous avons cherché à utiliser les RTF de manière optimale pour

augmenter la durée de survie de *D. sukii* lors d'un stockage au froid. Nous avons fait varier plusieurs paramètres : la température (15, 20, 25°C), la durée (30 min, 1, 2, 3 h), et la fréquence (toutes les 12, 24, 36 ou 48 h) des intervalles de température chaude chez deux lignées sauvages de *D. sukii*, à deux stades de développement (pupes et adultes). De manière générale, les RTF augmentaient la survie lors du stockage en comparaison aux températures froides constantes (TFC). La mortalité était la plus basse lorsque la température de récupération était de 20°C ou plus, la durée de l'intervalle égale à 2 h ou plus, et lorsque les fluctuations se produisaient fréquemment (toutes les 12 ou 24 h). L'utilisation d'un RTF optimal (20°C pendant 3 h toutes les 12 h) permettait de grandement abaisser la mortalité due au froid lors d'un stockage à long terme des adultes (jusqu'à 130 jours). L'énergie nécessaire à la réparation des dégâts causés par les températures froides pourrait conduire à des compromis impactant d'autres traits d'histoire de vie des insectes. Pourtant cette hypothèse n'a pas été clairement testée dans la littérature. Par conséquent, nous avons également testé les effets des RTF sur la valeur adaptative des drosophiles. Pour les adultes, la survie post-stockage n'était pas affectée par les RTF, tout comme la fécondité des femelles ou la capacité d'accouplement des mâles. Lorsque des pupes étaient stockées, la survie post-stockage et la compétitivité des mâles étaient altérées par les TFC mais pas par les RTF. Toutefois, la fécondité des femelles était plus faible après un stockage au stade pupes sous RTF en comparaison aux TFC, suggérant un compromis entre la réparation des dégâts provoqués par les températures froides et la production d'œufs. Cette étude fournit des informations détaillées concernant l'application et l'optimisation d'un protocole de stockage de *D. sukii* basée sur les RTF, qui pourrait s'avérer de grande utilité pour le biocontrôle de ce ravageur.

ORIGINAL ARTICLE

Effects of fluctuating thermal regimes on cold survival and life history traits of the spotted wing *Drosophila* (*Drosophila suzukii*)Thomas Enriquez , David Ruel*, Maryvonne Charrier  and Hervé Colinet 

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Abstract *Drosophila suzukii* is an invasive pest causing severe damages to a large panel of cultivated crops. To facilitate its biocontrol with strategies such as sterile or incompatible insect techniques, *D. suzukii* must be mass-produced and then stored and transported under low temperature. Prolonged cold exposure induces chill injuries that can be mitigated if the cold period is interrupted with short warming intervals, referred to as fluctuating thermal regimes (FTR). In this study, we tested how to optimally use FTR to extend the shelf life of *D. suzukii* under cold storage. Several FTR parameters were assessed: temperature (15, 20, 25 °C), duration (0.5, 1, 2, 3 h), and frequency (every 12, 24, 36, 48 h) of warming intervals, in two wild-type lines and in two developmental stages (pupae and adults). Generally, FTR improved cold storage tolerance with respect to constant low temperatures (CLT). Cold mortality was lower when recovery temperature was 20 °C or higher, when duration was 2 h per day or longer, and when warming interruptions occurred frequently (every 12 or 24 h). Applying an optimized FTR protocol to adults greatly reduced cold mortality over long-term storage (up to 130 d). Consequences of FTR on fitness-related traits were also investigated. For adults, poststorage survival was unaffected by FTR, as was the case for female fecundity and male mating capacity. On the other hand, when cold storage occurred at pupal stage, poststorage survival and male mating capacity were altered under CLT, but not under FTR. After storage of pupae, female fecundity was lower under FTR compared to CLT, suggesting an energy trade-off between repair of chill damages and egg production. This study provides detailed information on the application and optimization of an FTR-based protocol for cold storage of *D. suzukii* that could be useful for the biocontrol of this pest.

Key words cold storage; fluctuating thermal regimes; life-history traits; recovery; spotted wing drosophila

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Introduction

Insects exposed to stressful low temperature often exhibit high mortality or sublethal effects affecting their life history traits (Denlinger & Lee, 2010). To tolerate and survive deleterious conditions, insects can use behavioral strategies, like avoidance (Hawes *et al.*, 2008), or acclimation-related physiological adjustments, such as heat shock proteins synthesis or cryoprotectant accumulation (Clark & Worland, 2008; Denlinger & Lee, 2010; Colinet *et al.*, 2013). A growing number of studies

have shown that interrupting the exposure to constant low temperatures (CLT) with periodic warm phases (referred to as fluctuating thermal regimes, FTR) can mitigate, or even offset cold-induced mortality (see Colinet *et al.*, 2015b, 2018 for review). Although FTR treatments are not ecologically relevant, they may be used as an efficient protocol for prolonging insect survival during cold storage (Colinet & Hance, 2010; Rinehart *et al.*, 2011; Košťál *et al.*, 2016; Colinet *et al.*, 2018). Despite being artificial, FTR may trigger processes similar to naturally fluctuating temperatures; therefore, their study may also help understanding insect response to natural thermal variations. Insect's chill-injuries are linked to metal ions, water and metabolic homeostasis deregulations, as well as potential alterations of proteins and cell membranes (Košťál *et al.*, 2004; Denlinger & Lee, 2010; MacMillan & Sinclair, 2011). Many researchers have proposed that FTR benefits are due to physiological repair or recovery during recurrent warm pulses (Colinet *et al.*, 2018).

The present study focuses on the spotted wing drosophila, *Drosophila suzukii*, an alien species, originating from Southeast Asia, that has been introduced in both Europe and North America in 2008 (Hauser *et al.*, 2009; Raspi *et al.*, 2011; Calabria *et al.*, 2012) and that is now widely spread in these areas (Hauser, 2011; Cini *et al.*, 2012). This fly is a pest of soft fruits. Indeed, females oviposit in ripe fruits that larvae consume (Kanzawa, 1939; Mitsui *et al.*, 2006). Consequently, *D. suzukii* provokes important economic losses in a large range of cultivated crops (Goodhue *et al.*, 2011; Walsh *et al.*, 2011; Asplen *et al.*, 2015). To biologically control this pest, classical biocontrol, as well as sterile insect technique (SIT) and incompatible insect technique (IIT) are currently under deep investigation (Nikolouli *et al.*, 2018). These environment-friendly methods require recurrent inundative releases of massive numbers of sterile or incompatible insects. In this regard, a cost-effective mass-production needs to be developed. Low-temperature storage and cold treatment are integral part of mass-rearing and release protocols. Insects are frequently exposed to low temperature at several critical steps (e.g., egg collection, rearing, shipping, handling, and release), and these stressful treatments often cause loss of quality and/or mortality, which can seriously compromise the success of these programs (Mutika *et al.*, 2014). As *D. suzukii* is a chill-susceptible species (Kimura, 2004; Dalton *et al.*, 2011; Jakobs *et al.*, 2015; Plantamp *et al.*, 2016; Ryan *et al.*, 2016; Enriquez & Colinet, 2017), implementing cold storage protocols without loss of performance may be challenging. Consequently, applying FTR could be a suitable option to mitigate negative effects of cold storage.

With SIT or IIT, released flies have to compete with wild flies, and therefore, preserving their performances after cold storage is essential.

The first aim of this study was to optimize FTR protocol for short-term cold storage of *D. suzukii* adults and pupae by sequentially changing different parameters, such as temperature, duration and frequency of warming interruptions. It has previously been showed in other species that an increased recovery duration associated with optimal recovery temperature can nearly counterbalance chilling damages (Colinet *et al.*, 2011; Yocum *et al.*, 2012; Rinehart *et al.*, 2016). Increasing the frequency of warming intervals has also been associated with reduced mortality in various species (Colinet *et al.*, 2006; Yocum *et al.*, 2012). Hanč and Nedvěd (1999) showed, however, that cold survival does not necessarily increase linearly with recovery temperature or duration. Thus, it is important to explore a range of conditions to optimally use FTR. In the present study, we tested whether (i) beneficial effect of FTR increases with recovery temperature and duration, (ii) there is an optimal combination of recovery temperature \times duration above which no additional benefit is observed, and (iii) the more frequently insects have the opportunity to recover the better the cold survival.

Fluctuating thermal regime is thought to allow repair mechanisms, but these processes likely require energy, which would normally be allocated to other biological functions. Warming pulses under FTR are known to be associated with an overshoot in metabolic rate, which suggests a higher energy consumption under FTR than under CLT (Lalouette *et al.*, 2011; Yocum *et al.*, 2011; Boardman *et al.*, 2013). As a result, it can be assumed that physiological repair under FTR is related to fitness costs. There are only some few evidences for this in *Bactrocera latifrons* (Takano, 2014), where FTR treatment reduced the number of insects laying viable eggs. Fecundity was also reduced after fluctuating treatment in *Zeiraphera canadiensis* (Carroll & Quiring, 1993) and in *Ceratitidis capitata* (Basson *et al.*, 2012), but these treatments were not proper FTR (we refer to "proper" FTR as prolonged cold periods interrupted with recurrent short warming intervals). Fitness costs of FTR treatments thus remain poorly investigated (Colinet *et al.*, 2018). The second aim of this work was precisely to test the impact of CLT and FTR on several life history traits of *D. suzukii*. We hypothesized that (i) prolonged cold stress may induce latent damage that could manifest several days after the end of cold storage and that may express as late mortality, especially under CLT and (ii) higher energetic costs of FTR may translate into reduced reproductive traits (i.e., fecundity, mating capacity).

Materials and methods

Flies origin and rearing

To ensure that the effect of FTR on *D. suzukii* cold tolerance was robust and consistent, all experimentations were performed twice with two different wild-type lines. The “line 1” was a population from infested fruits collected from different locations in Trentino (Italia), and brought to the Vigalzano station of the Edmund Mach foundation (46.042574 N, 11.135245 E) in 2011. This line was sent to our laboratory (Rennes, France) in early 2016. Flies from the “line 2” were collected from infested blueberries and raspberries in Thorigné Fouillard, France (48.0616 N, -1.2387 E) in September 2016. Flies were reared in glass bottles (100 mL) and supplied *ad libitum* with artificial diet (agar: 15 g, sucrose: 50 g, organic carrot powder: 50 g, brewer yeast: 30 g, cornmeal: 20 g, kalmus: 8 g [Kalmus, 1943], Nipagin: 8 mL, water: 1 L). At least 15 bottles of 100–300 flies per line were used for continuous maintenance and emerging flies from different bottles were crossed at each generation to limit inbreeding. Bottles were placed in incubators (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan) under standard conditions: 25 °C, 65%–70% RH, and 12 L : 12 D. Pupae and adults randomly taken from the rearing stock were used in all experiments. Pupae were collected 48 h after pupation (i.e., corresponding to 8 d after egg laying at 25 °C). Adults used in all experiments were 4 or 5 d old when tested to limit age-related differences in stress tolerance (Colinet *et al.*, 2015a). Males were separated from females visually (with an aspirator) without CO₂ to avoid stress due to anesthesia (Colinet & Renault, 2012).

Survival to thermal treatments (experiments 1–3)

Figure 1A illustrates the experimental scheme for the optimization of FTR protocol for short-term cold storage of *D. suzukii*. Both thermal treatments (CLT and FTR) were applied to both lines and both stages (pupae and adults). Temperatures for CLT were 5 and 7.5 °C for pupae and 5 °C for adults. In all experiments (1–7), the thermal conditions used in the different treatments were controlled by programmed incubators (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan). In FTR, incubators took approximately 30 min to increase temperature from 5 or 7.5 °C to 15, 20, or 25 °C. In pupae, a preliminary test at 10 °C yielded to approximately 100 % survival after 15 d; therefore, this temperature was not used for subsequent experiments because flies were unaffected by cold. For the same reason, 7.5 °C was not

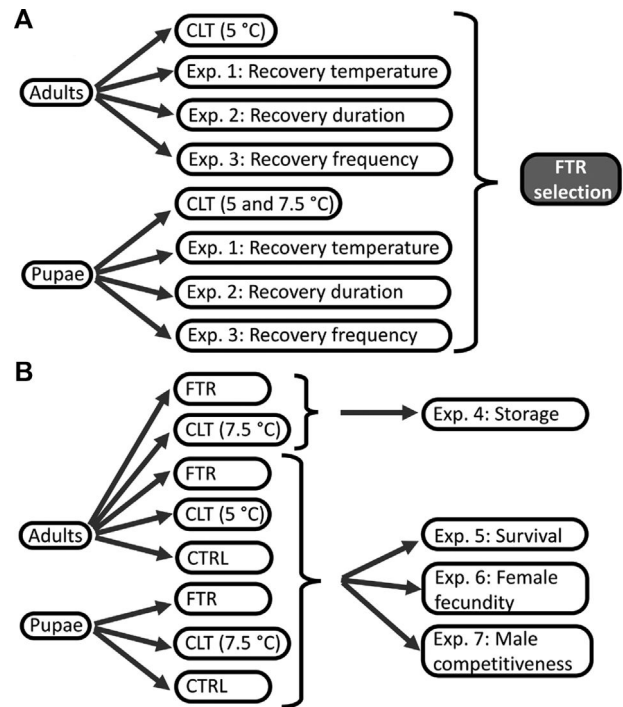


Fig. 1 Experimental plan. A: Survival assays. Pupae were exposed to constant low temperature (CLT) at 5 and 7.5 °C and adults were exposed to CLT at 5 °C. Cold exposures were also performed using fluctuating thermal regimes (FTR), where different recovery temperature (exp. 1), duration (exp. 2), and frequency (exp. 3) were tested. B: Impact of thermal treatments on cold storage tolerance and life history traits. Adults were exposed to either CLT (7.5 °C) or optimized FTR protocol, and long-term cold storage survival was followed for several weeks (exp. 4). Pupae and adults were subjected to optimized FTR or CLT or not exposed (control: CTRL). After treatments, post-storage survival (exp. 5), female fecundity (exp. 6), and male competitiveness (exp. 7) were assayed in both exposed adults and in adults emerging from exposed pupae.

used in adults. Indeed, in a previous work we showed that exposure to 7.5 °C only weakly affected adult’s survival (Enriquez & Colinet, 2017). For all assays, individuals were placed in vials and supplied with food. For pupae exposed to 5 °C, survival was monitored after 1, 2, 3, 4, 5, 6, or 7 d and for those exposed to 7.5 °C after 1, 2, 3, 4, 6, 8, or 10 d. For each time point, three tubes of 10 pupae were removed from cold condition and replaced at 25 °C. Survival was then scored as the number of emerged flies (partially emerged flies were considered as not emerged). For adults exposed to 5 °C, from both sexes separately, survival was monitored after 1, 2, 3, 5, 7, 9, or 12 d of cold exposure. For each time point, three tubes of 10 males and three tubes of 10 females were removed

from cold and replaced at 25 °C. Adult survival was assessed 24 h after flies were replaced to standard conditions.

For FTR, recovery temperature, duration and frequency were studied sequentially. For all FTR assays, the procedure was similar to that of CLT, except that the cold period was interrupted by recurrent recovery periods varying in (i) temperature: the cold period was interrupted daily by a 2 h break at 15, 20, or 25 °C (experiment 1); (ii) duration: the cold exposure was interrupted daily by a 20 °C break of 30 min, 1, 2, or 3 h (experiment 2); and (iii) frequency: the cold period was interrupted by 3 h breaks at 20 °C applied every 12, 24, 36, or 48 h (experiment 3). Each experiment was conducted on different cohorts from different generations (see Fig. 1A).

Long-term cold storage as adult (experiment 4)

After we selected the optimal parameters for short-term cold storage (experiments 1 to 3), an additional experiment was carried out to assess whether this optimized FTR protocol might be used to extend adult shelf life over much longer period (i.e., several weeks or months). For this purpose, adults (from the line 1 only) were exposed to either CLT at 7.5 °C or FTR where the cold period (7.5 °C) was interrupted twice a day by a break at 20 °C for 3 h (i.e., considered as optimal parameters). In this experiment, we choose 7.5 °C because this temperature is not too stressful for adults *D. sukii*, and therefore more appropriate for longer storage (Enriquez & Colinet, 2017). Males and females were separately placed in tubes of 20 flies and supplied with food. The experiment lasted 129 d. Approximately every 7 d, three tubes of 20 males and 20 females were removed from cold incubators, placed at 25 °C and survival was assessed after 24 h.

Poststorage survival (experiment 5)

Poststorage survival under standard conditions was monitored for 20 consecutive days following a short-term cold storage, in order to assess fitness consequences and putative latent damage resulting from cold treatment. Individuals were subjected to cold treatment either as pupae or as adult. Both lines were tested. The short-term cold treatment consisted of five consecutive days either under CLT (5 °C for adults and 7.5 °C for pupae) or under FTR, where the cold exposure (the same as under CLT) was interrupted every 12 h for 3 h at 20 °C. A control group (CTRL) was maintained under standard rearing condition at 25 °C (see Fig. 1B). For pupae, after 5 d under CLT or FTR, cold-exposed individuals were replaced to

standard conditions (25 °C) for emergence. Emerged males and females from CLT, FTR, and CTRL were then placed in food vials and their survival was monitored daily for 20 d. For adults, after 5 d under CLT or FTR, surviving flies were replaced to standard conditions in food vials and separated by sex. Survival was then monitored daily for 20 d. For each treatment, line and sex, five replicates of 10 flies were used. For both pupae and adult assays, food vials were changed every 2 d.

Female fecundity (experiment 6)

To assess the reproductive cost of a short-term cold treatment, fecundity was scored in cold-exposed females, as well as in females emerging from cold-exposed pupae. Both lines were tested. Pupae and females were exposed to CLT (7.5 °C), FTR (7.5 °C with 3 h breaks at 20 °C every 12 h), or unexposed to cold (CTRL) for five consecutive days (see Fig. 1B). Fecundity of 15 females from each treatment was then monitored daily for 15 consecutive days. Females were placed with two untreated males for the first 48 h of the experiment to allow mating. All females were kept individually into 50 mL falcon tubes under standard conditions. Tubes were placed vertically, plugs facing down. Plugs were filled with a medium composed of 3.5 g agar, 12.5 g sucrose, 20 g brewer yeast, 2 g Kalmus, and 2 mL Nipagin in 250 mL of water. Food plugs were changed daily, and eggs laid by females were counted under stereomicroscope.

Sexual competitiveness (experiment 7)

Male mating competitiveness was assessed following a short-term cold storage applied to both pupae and adults. All individuals used were isolated as pupae before emergence to avoid mating in order to get virgins. Both lines were tested. Males and pupae were exposed to CLT or FTR treatment or not cold exposed (CTRL) for five consecutive days, following the same method as described in “Female fecundity” section (Fig. 1B). Males were marked either on the left or the right wing to differentiate them during mating trials. To do so, males were briefly anesthetized under CO₂ (<1 min) and the last part of the marginal and submarginal wing cells was cut under stereomicroscope using forceps. A preliminary assay was performed to test a possible deleterious effect of the side of the cut wing on male competitiveness in CTRL flies, but no difference was observed between males marked on the right or the left wing (see results section). Within each treatment, half of the males were marked on the right wing, and the other half on the left wing. Mating competitiveness assays were

conducted 24 h after marking to avoid any deleterious effect due to anesthesia. All flies used in assays were marked (either on left or right wing) to avoid any confounding effect due to wing cut. In each trial, a piece of raspberry was placed at the bottom of a tube together with a virgin untreated female. Two marked virgin males from two different treatments were then simultaneously introduced in the vial for mating competition trials: (i) CTRL versus CLT (pupae: $n = 17$ replicates for the line 1 and 27 for the line 2; adults: $n = 30$ for the line 1, and 38 for the line 2), (ii) CTRL versus FTR (pupae: $n = 29$ replicates for the line 1 and 29 for the line 2; adults: $n = 23$ for the line 1, and 32 for the line 2). Tubes were then continuously inspected for 4 h. When a mating was observed, the treatment of the successful male was identified thanks to its wing cuts. Assays were conducted in the morning (from 8:00 to 12:00), corresponding to the period when *D. suzukii* is the most active (Hardeland, 1972; Evans *et al.*, 2017).

Statistical analyses

All analyzes were performed with R (R Core Team, 2016). Data from each survival experiment (exp. 1 to 3) and from long-term cold storage experiment (exp. 4) were analyzed using generalized linear models (GLMs) with logit link function for proportions outcome (i.e., number of dead/alive individuals per vial). For long-term cold storage (exp. 4), the 50% median lethal time (Lt_{50}) for each treatment and sex was calculated as follows (Venables & Ripley, 2002):

$$Lt_{50} = \frac{\text{logit}(0.5) - a}{b},$$

where a and b , respectively, correspond to the intercept and the slope of GLM prediction for each condition. GLM parameters were then resampled (1000 iterations, thanks to “arm” package; Gelman & Sue, 2014). Thereafter, the Lt_{50} values obtained from each condition were compared using a two-way ANOVA followed by Tukey’s *post hoc* tests (package “multcomp”; Hothorn *et al.*, 2008). For poststorage survival (exp. 5), mixed effects generalized models (GLMMs) with logit link function for proportions outcomes were applied. As the same flies within a vial were monitored every day, the vial identity was used as a random variable to account for repeated measures. Female fecundity (exp. 6) was analyzed using a GLMM following a Poisson error family, with a Log link function. As females were individually followed for 15 d, female identity was considered as a random variable. In all experiments, models were simplified by removing nonsignificant

interactions (Crawley, 2007). The effects of each variable and their interactions were analyzed via the analysis of deviance (“ANOVA” function in “car” package; Fox & Weisberg, 2011). Then, differences among thermal treatments were analyzed by comparing least-squares means using the “lsmeans” package (Russell, 2016). Finally, to help interpreting all the terms of models, effect plot function in the package “effects” (Fox, 2003) was used. The effect plots generated show the conditional coefficients (“marginal effects”) for all variables and interaction terms. Finally, data from competitiveness assays (exp. 7) were analyzed using exact binomial tests based on the null hypothesis that both competitors had equal probability of success ($P = 0.5$).

Results

For all the experiments, data from adults and pupae were analyzed separately. In order to facilitate the reading, we only provide in the main document a simplified version of the statistical outputs in Table 1, in which only the effect of the main variables of interest are presented. Comprehensive statistical outputs that show the significance of all model’s variables and all their interactions are available in Table S1, for all the experiments separately. Outcomes from effects plots illustrating the main effects and interactions are also available in supplementary figures for each experiment separately (Figs. S1–S11). Outcomes from multiple comparisons between thermal treatments are shown within these figures by different lettering (Figs. S1–S11). Furthermore, to simplify the main message, the graphical representations of models show results from both lines pooled, although both lines were always considered separately in all analyses. Graphical representations that differentiate both lines can still be found in supplementary figures (Fig. S12).

Recovery temperature (exp. 1)

Adult’s cold survival was significantly affected by all the main effects: sex, time, and thermal treatment (i.e., CLT or FTR at 15, 20, or 25 °C) (Table 1; Fig. 2A, B). Males had higher survival than females (Table 1; Figs. 2A, B, S1B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 2A, B, S1C). Adults survival was globally much higher under FTR than under CLT (Table 1; Fig. 2A, B), and *post hoc* comparisons showed that recovery temperature of 20 and 25 °C provided the highest survival (Fig. S1D). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 2A, B). In females, survival gradually increased

Table 1 Statistical outputs from GLMs and GLMMs. GLMs were used to analyze data from experiments 1, 2, 3, and 4: variation of recovery temperature, duration, frequency, and long-term storage. Data from experiment 5 and 6 (poststorage survival and female fecundity) were analyzed with GLMMs. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; NS: not significant. TRT stands for thermal treatment (i.e., CLT, FTR, and CTRL). The parameter “temp” refers to temperature of cold period (i.e., 5 or 7.5 °C for pupae). TRT: treatment (CLT: constant low temperature; FTR: fluctuating thermal regime; CTRL: control).

Experiment:	Exp. 1: Recovery temperature			Exp. 2: Recovery duration			Exp. 3: Recovery frequency			Exp. 4: Long-term cold storage			Exp. 5: Poststorage survival			Exp. 6: Females fecundity														
	Adults	Pupae		Adults	Pupae		Adults	Pupae		Adults	Pupae		Adults	Pupae		Adults	Pupae													
Parameter:	χ^2	df	P value	χ^2	df	P value	χ^2	df	P value	χ^2	df	P value	χ^2	df	P value	χ^2	df	P value												
Line	153.93	1	***	0.13	1	NS	21.73	1	***	131.3	1	***	134.52	1	***	40.53	1	***	516.14	1	***	334.54	1	***	2432.04	1	***			
Temp	NA	NA	***	190.38	1	***	NA	NA	***	460.13	1	***	NA	NA	***	249.14	1	***	NA	NA	***	NA	NA	***	NA	NA	***			
Sex	33.42	1	***	NA	NA	***	32.1	1	***	NA	NA	***	95.76	1	***	713.34	1	***	66.47	1	***	1.52	1	NS	1.82	1	NS			
Time	1077.69	1	***	581.84	1	***	1102.96	1	***	1167.05	1	***	947.14	1	***	519.95	1	***	519.95	1	***	293.47	1	***	145.33	1	***	286.88	1	***
TRT	350.52	3	***	166.45	3	***	1486.87	4	***	221.24	4	***	647.22	4	***	254.67	4	***	303.67	1	***	32.6	2	***	962.22	2	***	2072.35	2	***
TRT:line	44.13	3	***	49.17	3	***	43.38	4	***	2.17	4	NS	12.39	4	*	25.74	4	***	NA	NA	***	1.65	2	NS	33.38	2	***	666.81	2	***
TRT:temp	NA	NA	***	19.41	3	***	NA	NA	***	27.99	4	***	87.98	4	***	22.31	4	***	8.38	1	**	6.39	2	*	25.88	2	***	NA	NA	***
TRT:sex	40.98	3	***	NA	NA	***	23.9	4	***	NA	NA	***	505.21	4	***	237.39	4	***	0.41	1	NS	9.06	2	*	19.09	2	***	652.6	2	***
Time:TRT	408.1	3	***	221.14	3	***	291.13	4	***	135.6	4	***	505.21	4	***	237.39	4	***	0.41	1	NS	9.06	2	*	19.09	2	***	652.6	2	***

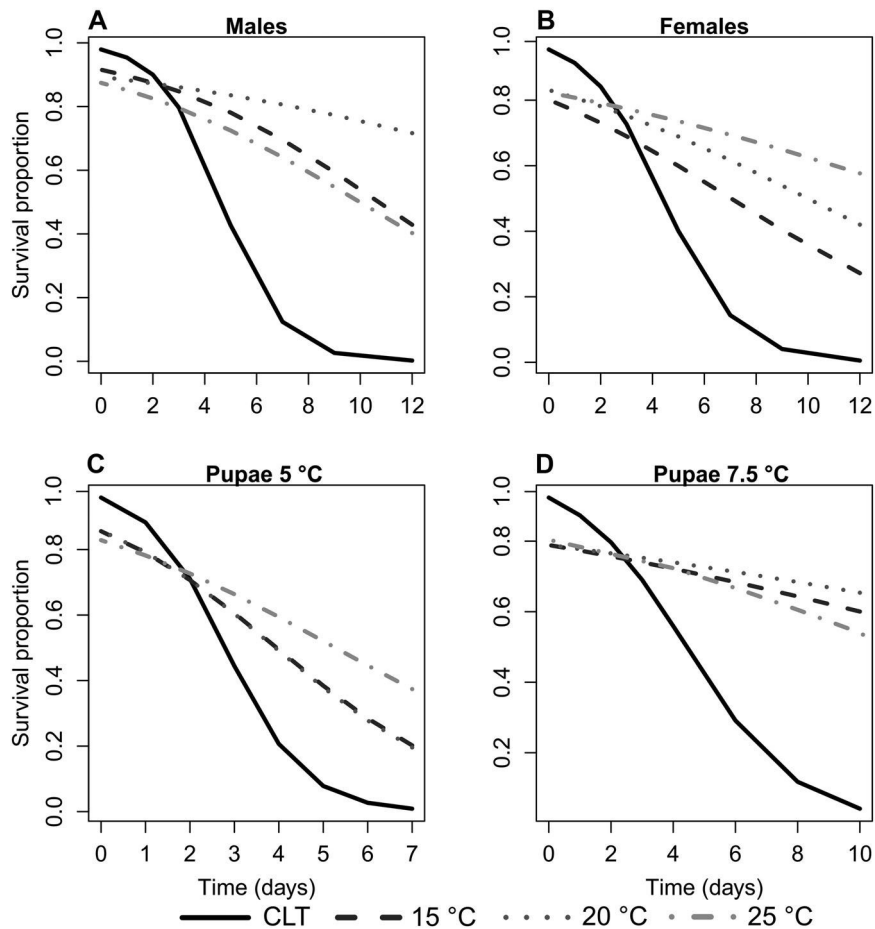


Fig. 2 Survival responses to changes in recovery temperature. Solid black curve: insects subjected to constant low temperature (CLT). Dashed gray curves: FTR where the cold exposure was interrupted by a daily recovery period of 2 h at 15, 20 or 25 °C. The curves correspond to model predictions (Binomial GLM, logit link function). A: males; B: females; C: pupae exposed to 5 °C; D: pupae exposed to 7.5 °C.

with recovery temperature, while in males, survival was the highest with a recovery temperature of 20 °C (Table 1; Figs. 2A, B, S1I). Finally, temporal decrease in survival was much faster under CLT than under FTR treatments (Table 1; Figs. 2A, B, S1J).

Cold survival of pupae was significantly affected by temperature, time, and thermal treatment (Table 1; Fig. 2C, D). Survival was higher when the cold period was 7.5 compared to 5 °C (Table 1; Figs. 2C, D, S2B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 2C, D, S2C). Pupal survival was globally much higher under FTR than under CLT (Table 1; Fig. 2C, D), and *post hoc* comparisons showed that all recovery temperatures were equivalent (Fig. S2D). The effect of thermal treatment also interacted with temperature and time (Table 1; Fig. 2C, D). When the cold period was

5 °C, pupal survival under both FTR and CLT was much lower than when it was 7.5 °C (Table 1; Figs. 2C, D, S2I). Finally, temporal decrease in pupal survival was much faster under CLT than under FTR treatments (Table 1; Figs. 2C, D, S2J).

Recovery duration (exp. 2)

Cold survival of adults was significantly affected by all the main effects: sex, time, and thermal treatment (i.e., CLT or FTR for 30 min, 1, 2 or 3 h) (Table 1; Fig. 3A, B). Males had higher survival than females (Table 1; Figs. 3A, B, S3B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 3A, B, S3C). Adults survival was globally much higher under FTR than under CLT (Table 1; Fig. 3A, B), and *post*

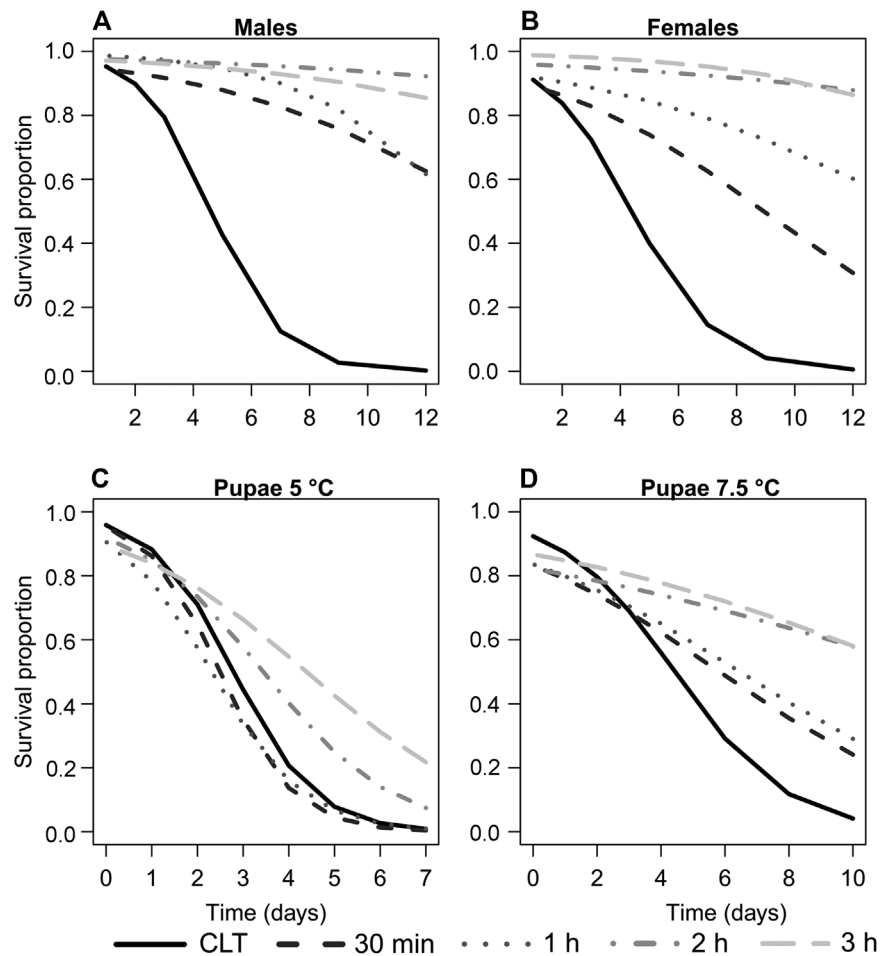


Fig. 3 Survival responses to changes in recovery duration. Solid black curve: Insects subjected to constant low temperature (CLT). Dashed gray curves: FTR where the cold exposure was interrupted by a daily recovery period at 20 °C for 30 min, 1, 2, or 3 h. The curves correspond to model predictions (Binomial GLM, logit link function). A: males; B: females; C: pupae exposed to 5 °C; D: pupae exposed to 7.5 °C.

hoc comparisons showed that recovery duration of 2 and 3 h provided higher survival than duration of 0.5 and 1 h (Fig. S3D). It should be noted that with FTR treatments allowing 2 and 3 h recovery, adult cold mortality was nearly fully compensated (Figs. 3A, B). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 3A, B). In females, survival gradually increased with recovery duration, while in males, survival was highest with 2 h of recovery (Table 1; Figs. 3A, B, S3I). Finally, temporal decrease in survival was much faster under CLT than under FTR treatments. Among FTR conditions, survival decreased with time more slowly when recovery duration was 2 and 3 h (Table 1; Figs. 3A, B, S3J).

Cold survival of pupae was significantly affected by temperature, time and thermal treatment (Table 1; Fig.

3C, D). Survival was higher when the cold period was 7.5 °C compared to 5 °C (Table 1; Figs. 3C, D, S4B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 3C, D, S4C). Pupal survival was globally much higher under FTR than under CLT (Table 1; Fig. 3C, D), and *post hoc* comparisons showed that recovery duration had marked effect on pupal survival with 3 h being the best, followed by 2 h, and durations of 1 or 0.5 h were not different from CLT (Fig. S4D). The effect of thermal treatment also interacted with temperature and time (Table 1; Fig. 3C, D). When the cold period was 5 °C, pupal survival under both FTR and CLT was much lower than when it was 7.5 °C (Table 1; Figs. 3C, D, S4I). Finally, temporal decrease in pupal survival was much faster under CLT than under FTR treatments (Table 1; Figs. 3C, D, S4J).

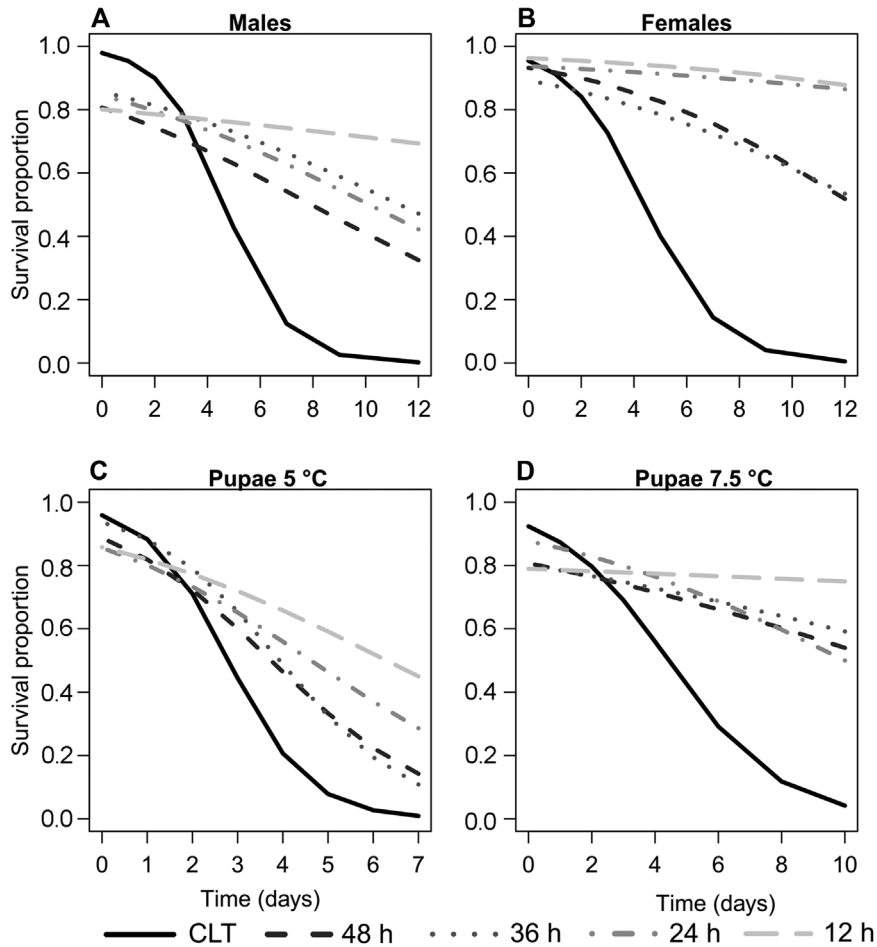


Fig. 4 Survival responses to changes in recovery frequency. Solid black curve: insects subjected to constant low temperature (CLT). Dashed gray curves: FTR where the cold exposure was interrupted by a 3 h recovery period at 20 °C, applied every 48, 36, 24, or 12 h. The curves correspond to model predictions (binomial GLM, logit link function). A: males; B: females; C: pupae exposed to 5 °C; D: pupae exposed to 7.5 °C.

Recovery frequency (exp. 3)

Cold survival of adults was significantly affected by all the main effects: sex, time, and thermal treatment (i.e., CLT or FTR every 12, 24, 36, or 48 h) (Table 1; Fig. 4A, B). Males had lower survival than females (Table 1; Fig. 4A, B, S5B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 4A, B, S5C). Adults survival was globally much higher under FTR than under CLT (Table 1; Fig. 4A, B), and *post hoc* comparisons showed that recovery frequency of 12 and 24 h provided highest survival, followed by 36 and 48 h (Fig. S5D). With FTR treatments cycling every 12 h, cold mortality was nearly fully compensated, especially in females (Fig. 4A, B). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 4A, B). In females, survival

was clearly higher with recovery frequencies of 12 and 24 h, while in males, effects were less contrasted (Table 1; Figs. 4A, B, S5I). Finally, temporal decrease in survival was much more rapid under CLT than under FTR treatments, and among FTR conditions, survival decreased with time more slowly when recovery periods were frequent (Table 1; Figs. 4A, B, S5J).

Cold survival of pupae was significantly affected by temperature, time and thermal treatment (Table 1; Fig. 4C, D). Survival was higher when the cold period was 7.5 compared to 5 °C (Table 1; Figs. 4C, D, S6B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 4C, D, S6C). Pupal survival was globally much higher under FTR than under CLT (Table 1; Fig. 4C, D), and *post hoc* comparisons showed that recovery frequency of 12 h provided highest survival, followed

by 24, 36 and then 48 h (Fig. S6D). The effect of thermal treatment also interacted with temperature and time (Table 1; Fig. 4C, D). When the cold period was 5 °C, pupal survival under both FTR and CLT was much lower than when it was 7.5 °C (Table 1; Figs. 4C, D, S6I). Finally, temporal decrease in pupal survival was much faster under CLT than under FTR treatments (Table 1; Figs. 4C, D, S6J).

Long-term cold storage of adults (exp. 4)

Fig. 5 shows the results from long-term cold storage of adults exposed to two thermal treatments: CLT (7.5 °C) or FTR (7.5 °C alternating with breaks of 20 °C for 3 h occurring twice a day). Cold survival was significantly affected by sex, time, and thermal treatment (Table 1; Fig. 5A, B). Females were globally more cold tolerant than males (Table 1; Figs. 5A, B, S7A). Cold survival decreased with increasing time of exposure (Table 1; Figs. 5A, B, S7B). Survival was higher under FTR than under CLT (Table 1; Figs. 5A, B, S7C). The effect of thermal treatment also interacted with sex but not with time (Table 1; Fig. 5A, B). Males benefited more from FTR than females (Table 1; Figs. 5A, B, S7E). Temporal decrease in survival did not differ among treatments (Table 1; Figs. 5A, B, S7F). Under CLT, males and females Lt_{50} values were, respectively, 26.14 and 54.36 d, while under FTR, they were, respectively, 45.61 and 118.32 d (Fig. 5C). Lt_{50} values were all significantly different ($F = 50.828$, $df = 3$, $P < 0.001$; all Tukey's P values < 0.001).

Poststorage survival (exp. 5)

Survival was recorded for 20 d (at 25 °C) following three treatments: 5-d cold exposure (CLT or FTR), or no cold exposure (CTRL). For adults, survival was significantly affected by time and thermal treatment, but not by sex (Table 1; Fig. 6A, B). Survival decreased with time (Table 1; Figs. 6A, B, S8C). Overall, CLT showed the lowest survival rate, followed by CTRL and then FTR (Table 1; Figs. 6A, B, S8D). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 6A, B). The effect of thermal treatment was not pronounced in females (because survival was globally high), while in males, positive effect of FTR was more evident (Table 1; Figs. 6A, B, S8I). Temporal decrease in survival varied according to treatments and was faster under CLT than under FTR or CLTR (Table 1; Figs. 6A, B, S8J).

When exposure occurred at pupal stage, subsequent 20-d adult survival was affected by time and thermal treatment but not by sex (Table 1; Fig. 6E–H). Survival

decreased with time (Table 1; Figs. 6C, D, S9C). CLT showed the lowest survival, followed by FTR, and then CTRL (Table 1; Figs. 6C, D, S9D). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 6C, D). Survival under FTR did not differ from CTRL in females but was lower than CTRL in males (Table 1; Figs. 6C, D, S9I). Temporal decrease in survival differed among treatments, with CLT showing the fastest decrease (Table 1; Figs. 6C, D, S9J).

Female fecundity (exp. 6)

Cumulated fecundity (over 15 d) of females that were cold exposed as adults varied with time and thermal treatment (Table 1; Fig. 7A). Cumulated fecundity increased with time (Table 1; Figs. 7A, S10B). Females exposed to CLT produced the lowest number of eggs, followed by females exposed to FTR, then, unexposed CTRL females were the most productive (Table 1; Figs. 7A, S10C). The effect of thermal treatment on fecundity also interacted with time (Table 1; Fig. 7A). Temporal egg-laying patterns differed among treatments: after FTR or CLT treatment, the beginning of the egg-laying phase was delayed in comparison with the CTRL group (Table 1; Figs. 7A, S10F).

Cumulated fecundity of females emerging from cold-exposed pupae was affected by time and thermal treatment (Table 1; Fig. 7B). Cumulated fecundity increased with time (Table 1; Figs. 7B, S11B). Pupae exposed to FTR resulted in the lowest fecundity, followed by CLT then CTRL (Table 1; Figs. 7B, S11C). The effect of thermal treatment on fecundity also interacted with time (Table 1; Fig. 7B). Finally, temporal egg-laying patterns differed among treatments: start of the egg-laying phase was slightly delayed under FTR in comparison with CTRL (Table 1; Figs. 7B, S11F).

Mating competitiveness (exp. 7)

Outcomes of male mating competitiveness are shown in Fig. 8. First, effect of the side of the wing cut on mating success in CTRL flies was tested (using exact binomial tests) and no effect was found (left vs. right, $P = 0.56$). Next, we tested effect of cold treatments on male mating success. Males cold-treated as adult, whether under CLT or FTR, showed similar mating success as CTRL males (CTRL vs. FTR, $P = 1$; CTRL vs. CLT, $P = 0.90$). The only significant effect detected was in males subjected to CLT as pupae that had lower mating success than CTRL males (CTRL vs. CLT, $P < 0.05$). Males exposed to FTR

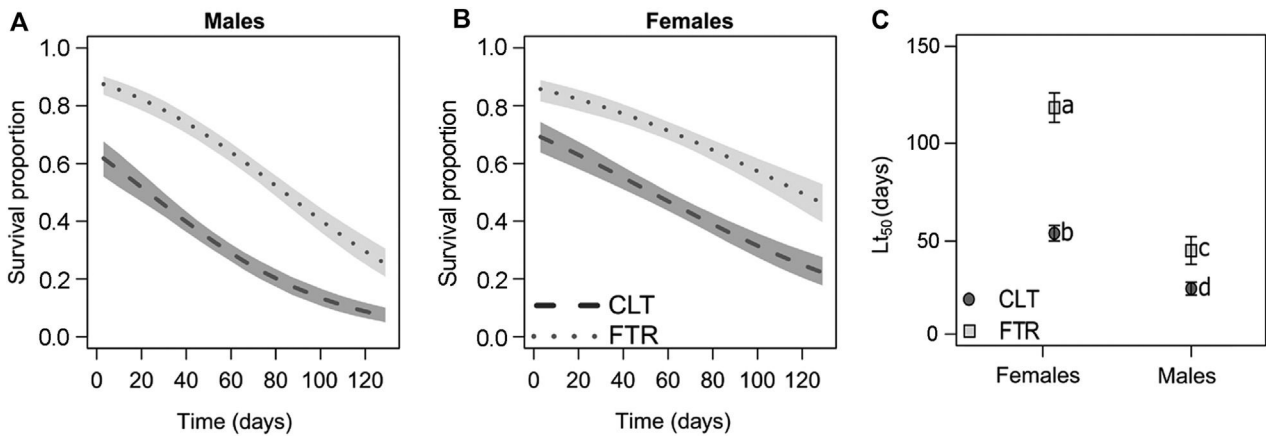


Fig. 5 Survival responses to long-term cold storage. Dashed gray curve: CLT (7.5 °C); dotted gray curve: FTR where the cold exposure was interrupted by breaks of 3 h at 20 °C every 12 h. The curves correspond to model predictions (binomial GLM, logit link function), and shaded areas to 95% interval confidence. A: males; B: females. C: estimated Lt_{50} values from GLMs. Symbols with different letters are significantly different ($P < 0.001$).

as pupae had similar mating success as CTRL males (CTRL vs. FTR, $P = 0.35$).

Discussion

FTR effect on cold survival

In this study, we analyzed cold storage of *D. suzukii* under CLT and FTR, and explored consequences on survival and fitness-related traits. Concerning effects of FTR on survival, we hypothesized that beneficial effect of FTR should increase with recovery temperature, duration, and frequency, and that there should be an optimal combination of recovery temperature \times duration above which no additional benefit is observed. Most of the tested FTR treatments led to significant reduction in cold-induced mortality compared to CLT, corroborating earlier observations about the positive effect of FTR (e.g., Renault *et al.*, 2004; Colinet *et al.*, 2006; Košťál *et al.*, 2007; Javal *et al.*, 2016; Torson *et al.*, 2017). Although, the benefits of FTR were of different magnitude depending on the various experimental conditions. Beneficial effects of FTR were robust, as it was consistently observed in both lines, sexes and life stages. Other studies showed that FTR has positive effects on pupal survival (Colinet *et al.*, 2006; Dollo *et al.*, 2010; Rinehart *et al.*, 2011; Yocum *et al.*, 2012, 2016), but in *D. melanogaster* a positive response of FTR at pupal stage was not clearly observed (Javal *et al.*, 2016). The temperatures tested by Javal *et al.* (2016) were lower than 5 °C, and at these temperatures, chilling damage might be harder to recover.

We aimed to identify an optimal FTR protocol for short- or long-term cold storage from the best combination of recovery temperature, duration and frequency in both pupae and adults. Among the three recovery temperatures tested, 20 and 25 °C provided roughly similar benefits. Using 15 °C as a recovery temperature also allowed insects to survive cold storage longer than their counterparts exposed to CLT, but benefits were slightly less evident than at 20 and 25 °C. Previous studies have found that increasing FTR recovery temperature results in better survival (Nedvěď *et al.*, 1998; Renault *et al.*, 2004; Colinet *et al.*, 2011; Yocum *et al.*, 2012), but we found that an increase of 5 °C (from 20 to 25 °C) did not significantly promoted survival. Furthermore, a too high recovery temperature may eventually become deleterious (Hanč & Nedvěď, 1999). Moreover, when the recovery temperature was set at 25 °C, during decreasing temperature phases, relative humidity could reach 100%, resulting in condensation of water inside incubator and within vials. Condensation can be deleterious for flies, as they may easily get stuck on droplets and died. Moreover, fungal development is favored by high relative humidity, which may compromise insect storage (Colinet *et al.*, 2018). Consequently, only 20 °C was conserved for the follow-up experiments. Increasing the recovery duration and frequency resulted in a gradual decrease in mortality. In most cases, the longer and the most frequent the recovery duration, the lower the mortality, in both pupae and adults. This is consistent with earlier data on other insects (Hanč & Nedvěď, 1999; Colinet *et al.*, 2006; Yocum *et al.*, 2012). Interestingly, even a very short recovery duration (30 min) decreased cold-induced mortality, suggesting

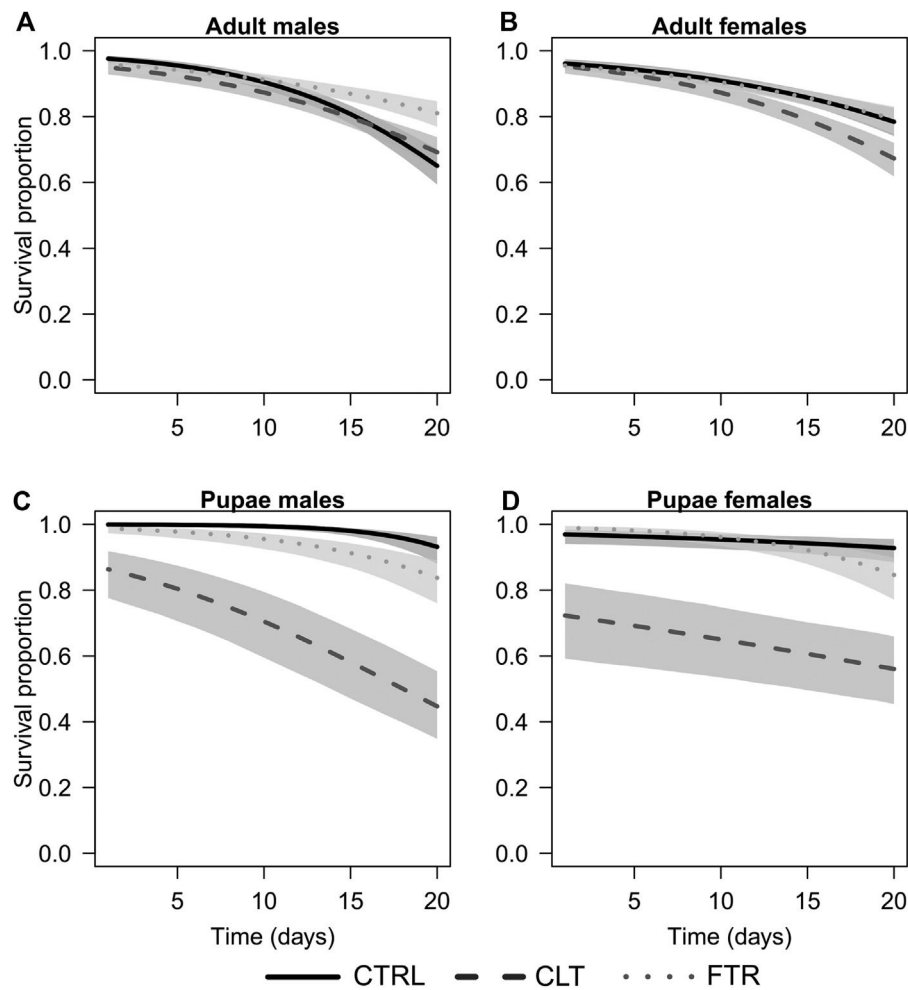


Fig. 6 Survival at 25 °C subsequent to a short cold-exposure under different regimes. Solid black curve: control (CTRL: 25 °C for 5 d); dashed gray curve: CLT applied to adults (5 °C for 5 d) and to pupae (7.5 °C for 5 d); dotted gray curve: FTR where the cold exposure was interrupted by breaks of 3 h at 20 °C every 12 h. FTR was applied to adults and pupae for 5 d. After exposure to CTRL, CLT, and FTR for 5 d, survival was monitored for 20 d under standard conditions. The curves correspond to model predictions (binomial GLMM, logit link function), and shaded areas to 95% interval confidence. A: adult males; B: adult females; C: males from cold-exposed pupae; D: females from cold-exposed pupae.

that, as many other species, *D. sukikii* has the capacity to quickly recover from cold stress when temperature turns favorable.

By systematically changing FTR parameters, we could define what we consider to be the optimal settings for *D. sukikii*: interruption of the cold period every 12 h by warming intervals at 20 °C for 3 h. This optimized treatment clearly allowed adults and pupae to remain alive at cold during a much longer period than insects exposed to CLT. When applied to adults, these optimal FTR settings almost doubled Lt_{50} values reaching 54 d in males and 118 d in females. Maximum longevity of *D. sukikii* under laboratory standard conditions does not exceed 35 d at

25 °C (Tochen *et al.*, 2014; Kim *et al.*, 2015). In this regard, the application of FTR could also be used as life-span extension protocol to maintain lines of interest, such as those used in SIT and IIT programs, with a low impact on fly's survival. In addition, this FTR protocol offers real opportunity for short-term cold storage of pupae that can be safely conserved for at least 10 d.

Impact of FTR on life history traits

The generally accepted explanation for the promoting effects of FTR is that warm interruptions allow repair

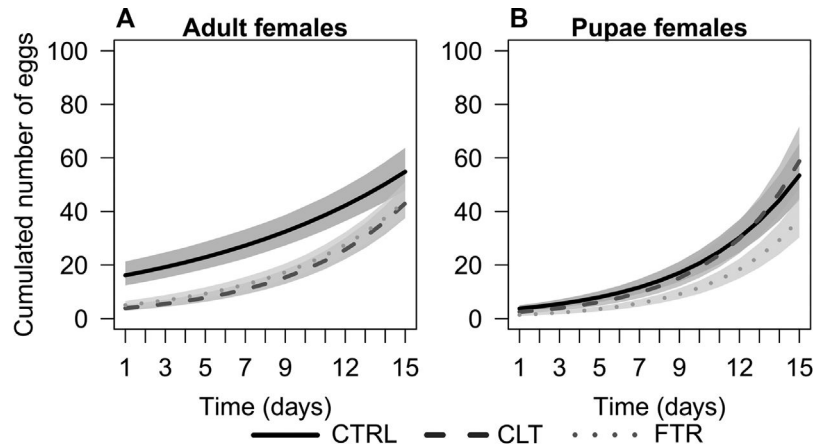


Fig. 7 Fifteen-day cumulated fecundity subsequent to a short cold-exposure under different regimes. Solid black curve: control (CTRL: 25 °C for 5 d); dashed gray curve: CLT applied to adults (5 °C for 5 d) and to pupae (7.5 °C for 5 d); dotted gray curve: FTR where the cold exposure was interrupted by breaks of 3 h at 20 °C every 12 h. FTR was applied to adults and pupae for 5 d. After exposure to CTRL, CLT, and FTR for 5 d, the fecundity was monitored for 15 d under standard conditions. The curves correspond to model predictions (Poisson GLMM, log link function), and shaded areas to 95% interval confidence. A: adult females; B: females from cold-exposed pupae.

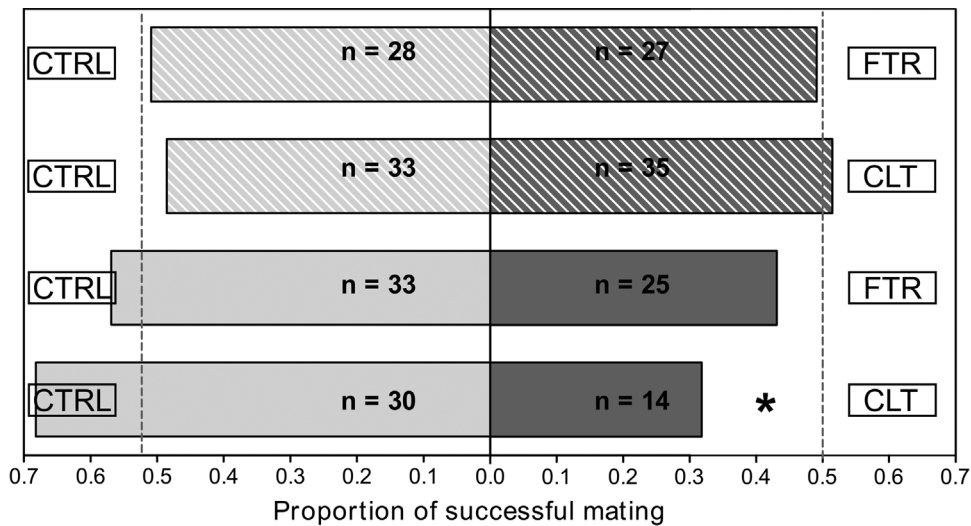


Fig. 8 Male competitiveness subsequent to a short cold-exposure under different regimes. Light-gray bars: control males (CTRL: 25 °C for 5 d); dark-gray bars: males exposed to CLT (5 or 7.5 °C for 5 d in adults and pupae, respectively) and males exposed to FTR for 5 d (cold interrupted by breaks of 3 h at 20 °C every 12 h). Full bars correspond to individuals exposed to thermal treatments at pupal stage, and dashed bars correspond to individuals exposed as adults. After exposure to CTRL, CLT, and FTR for 5 d, male competitiveness was monitored in mating assays. Numbers of successful males are indicated inside their respective bars. The sign (*) corresponds to $P < 0.05$.

of chilling-injuries that otherwise accumulate under CLT (Renault *et al.*, 2004; Košťál *et al.*, 2007; Colinet *et al.*, 2018). Several repair mechanisms have been supposed to be involved, such as reestablishment of ion homeostasis (Košťál *et al.*, 2007), restoration of membrane lipids composition (Colinet *et al.*, 2016), activation of antioxidant system (Lalouette *et al.*, 2011; Torson *et al.*, 2017),

accumulation of cryoprotectants, and production of heat shock proteins (Pio & Baust, 1988; Colinet *et al.*, 2007; Lalouette *et al.*, 2007; Boardman *et al.*, 2013). These adjustments are probably associated with energy cost for the organisms (Colinet *et al.*, 2018), consequently, the second aim of this work was to explore fitness consequences of cold storage under CLT versus FTR, assuming that

CLT would induce deleterious effects that would carry over into adult life, and that FTR would generate cost that could alter life history traits.

Survival after cold treatment Survival of adults was followed for 20 consecutive days after a cold exposure of 5 d (CLT vs. FTR) applied either at the adult or pupal stage, and this was compared to patterns of flies kept at 25 °C (CTRL). Data revealed that CLT markedly decreased subsequent adult survival compared to unexposed CTRL or FTR-exposed flies, especially when individuals were treated at the pupal stage. By contrast, flies' survival was largely superior under FTR than under CLT, especially when FTR was applied to adults. When applied to pupae, benefits of FTR were slightly less evident. It is possible that FTR applied to adults allows efficient repair of chilling injuries, but when applied to pupae, cold damage may not be fully recovered and carry-over in the next stage. Our results show that pupae are globally more cold susceptible than adults. Previous works on *D. sukikii* have highlighted these differences (Dalton *et al.*, 2011; Ryan *et al.*, 2016; Enriquez & Colinet, 2017). We found that exposure to stressing CLT in pupae had lasting effects on the adults emerging from these pupae. Metamorphosis is ongoing during pupal stage and a prolonged cold stress during this critical phase might induce latent damages with a knock-on effect in adults. In the butterfly *Bicyclus anynana*, heat stress experienced early in life carried over to later stages, reducing subsequent fitness (Klockmann *et al.*, 2017). In *Plutella xylostella*, heat injury in eggs and 3rd-instar larvae also carried over to the adult stage and led to a more rapid rate of egg laying (Zhang *et al.*, 2015). The impact of cold stress on developing stages and the resulting consequences on adult survival and fitness remain poorly documented. Here, we provide evidence of a carry-over effect on adult's survival, resulting from cold stress at pupal stage, particularly when pupae were exposed to prolonged cold stress without opportunity to recover.

FTR impact on female fecundity Effects of FTR on fecundity have been poorly documented. In the present study, cold-exposed females, under both FTR and CLT, showed a delayed and reduced cumulated egg production compared to unexposed CTRL flies. This result suggests a negative impact of low temperature on subsequent reproductive potential. Stressful temperature, like heat stress, can alter egg development and can induce oviposition delays due to hormonal imbalance (Gruntenko *et al.*, 2003). Cold stress may also cause damages to diverse tissues in insects, leading to impairment of reproductive abilities (Rinehart *et al.*, 2000) or delayed ovarian development (Jones & Kunz, 1998). These phenomena may have led to

the observed decrease in fecundity. We also observed that *D. sukikii* flies cold-treated as pupae showed lower fecundity than controls, particularly when exposed to FTR. Ismail *et al.* (2010) did not observe any fecundity decrease after FTR exposition in *Aphidius ervi* females. Likewise, Murdoch *et al.* (2013) tested the effect of warming interruptions applied once or thrice during a cold storage period of 8-week and found no differences between constant or fluctuating storage on fecundity of the leek moth *Diadromus pulchellus*. Allocation of limited energy often results in life-history trade-offs, for instance between reproduction and longevity (Zera & Harshman, 2001; Attisano *et al.*, 2012). Contrary to CLT-exposed pupae, we found no sign for decreased longevity (i.e., 20 d survival poststress) of FTR-exposed pupae in comparison with CTRL pupae. On the other hand, we found evidence for reduced egg production in females exposed at pupal stage to FTR compared to CTRL, but not in pupae exposed to CLT. Energy used for repair mechanisms under FTR might have led to this reduced egg production.

Mating competitiveness Concerning sexual competitiveness, when males were subjected to CLT or FTR as adults, no difference was observed with control. However, when cold treatment was applied to pupae, FTR males showed similar mating performance as control males, but CLT males showed a decreased mating success. Sub- and supra-optimal temperatures decrease sperm production and viability, and can cause sterility (Rinehart *et al.*, 2000; Araripe *et al.*, 2004; David *et al.*, 2005; Porcelli *et al.*, 2017). In the parasitoid wasp *Dinarmus basalis*, a cold stress during pupal development had detrimental effects on eclosion of pupae and males showed a reduced sperm stock at emergence. These cold-stressed males were at a disadvantage in accessing females and inseminated fewer females than control wasps (Lacoume *et al.*, 2007). Although we did not check the sperm production neither the sperm viability of cold-exposed *D. sukikii* males, we may hypothesize that CLT (i.e., the most stressful treatment according to our data) may have induced an alteration of spermatogenesis, which may have influenced mating behavior and male's success. Colinet and Hance (2009) also showed that exposing parasitoids pupae to cold storage reduced male's mating success when exposed to CLT, but not to FTR. CLT-exposed wasps had altered mobility and velocity, probably due to muscles impairments. In *Drosophila* flies, male courtship success is correlated with running speed and success in aggressive interactions with competitive males (Partridge & Farquhar, 1983). Therefore, we can also assume that a reduction in mating performances of *D. sukikii* males exposed to CLT may be, at least in part, a consequence of a reduced mobility.

Several studies that looked at the cold stress effect on pupae observed pharate adults unable to fully emerge from puparium (Lacoume *et al.*, 2007). This phenomenon has been attributed to muscle contraction deficiency (Yocum *et al.*, 1994; Kelty *et al.*, 1996), resulting from neuromuscular dysfunctions due to low temperature (Košťál *et al.*, 2004; 2007). Since FTR allows the restoration and maintenance of ion homeostasis (Košťál *et al.*, 2007, 2016), FTR-exposed flies were possibly less subjected to neuromuscular and mobility disfunctions.

Concluding remarks

The present work conclusively shows that storage at 5 or 7.5 °C interrupted by recovery periods at 20 °C for 3 h every 12 h is particularly appropriate for storage of adults. Pupae were globally more sensitive to cold than adults, but FTR allowed this stage to be stored with minimum impact on survival for approximately 10 d. Even if 7.5 °C under FTR seems promising for relatively short storage durations of pupae, caution needs to be taken with longer periods, as development can still proceed under these conditions and may lead to undesirable emergence during cold storage. In the present study, we focused on thermal-related parameters of FTR, but other parameters could be important for cold storage, such as relative humidity, which can affect thermal tolerance of insects, including *D. suzukii* (Boardman *et al.*, 2013; Enriquez & Colinet, 2017; Eben *et al.*, 2018). Even if FTR offer promising results in laboratory scaled experiments, implementation of such technique in industrial scale may be costly and challenging (Colinet *et al.*, 2018). Works are still required to study and to adapt FTR to face these constraints. Finally, from a fundamental aspect, the bioenergetics of FTR remain poorly explored, and future studies should aim to determine whether or not the energy depleted during cold storage drives life-history trade-offs.

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Disclosure

The authors declare there are no competing interests.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Effect plots from GLM: temperature variation of FTR on adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S1D correspond to statistical differences between treatments (least-square means, $P < 0.05$)

Fig. S2 Effect plots from GLM: temperature variation of FTR on pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line, and their interactions. Different letters on Fig. S2D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S3 Effect plots from GLM: duration variation of FTR on adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S3D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S4 Effect plots from GLM: duration variation of FTR on pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model

as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line, and their interactions. Different letters on Fig. S4D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S5 Effect plots from GLM: frequency variation of FTR on adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S5D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S6. Effect plots from GLM: frequency variation of FTR on pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line, and their interactions. Different letters on Fig. S6D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S7 Effect plots from GLM: medium-term cold storage as adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, and their interactions.

Fig. S8 Effect plots from GLMM: poststorage survival of adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S8D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S9 Effect plots from GLMM: poststorage survival of pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model

as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S9D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S10 Effect plots from GLMM: Female fecundity of adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), line, and their interactions. Different letters on Fig. S10C correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S11 Effect plots from GLMM: Female fecundity of pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), line and their interactions. Different letters on Fig. S11C correspond to statistical differences between treatments (least-squares means, $P < 0.05$).

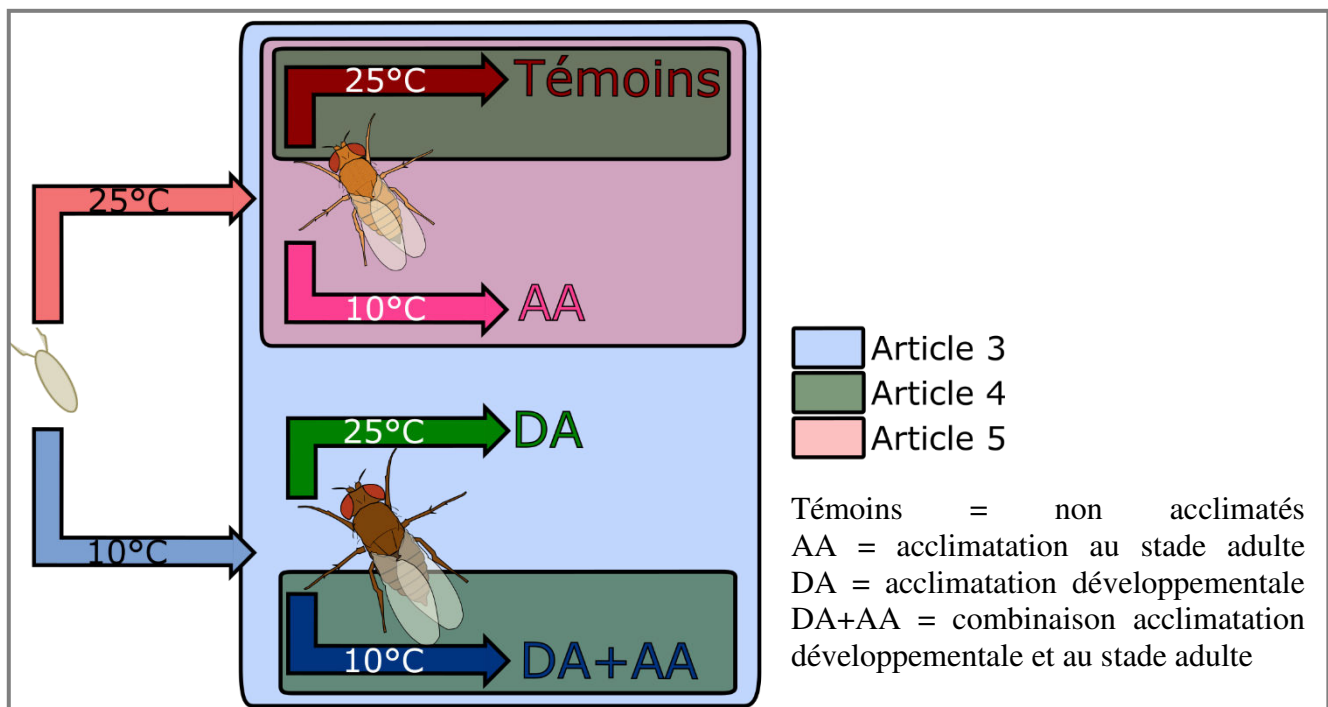
Fig. S12 Results from experiments 1 to 7 differentiate between lines 1 and 2. Each figure is associated with its caption.

Table S1: Detailed statistics from GLMs and GLMMs. Datasets from variation of temperature, duration, and frequency of the recovery period and long-term storage GLMs were used. Poststorage survival and female fecundity datasets were analyzed with GLMMs. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS: not significant. TRT stands for thermal treatment (i.e., for experiments 1 to 4: CLT vs. FTR, and for experiments 5 and 6: CTRL vs. FTR and CLT). The symbol “/” indicate parameters not considered in the experiments and the sign “–” is used for data not included in the statistical analysis. TRT: treatment (CLT: constant low temperature; FTR: fluctuating thermal regime; CTRL: control).

Chapitre 3 - Mécanismes sous-jacents à la plasticité de la thermotolérance de *Drosophila suzukii*

Contexte et objectif du chapitre :

La plasticité phénotypique est un facteur déterminant dans le succès des invasions biologique (voir partie **I.8. de l'introduction**). *Drosophila suzukii* est une espèce intolérante au froid (**chapitre 1**), pourtant elle est capable de survivre aux températures hivernales en Europe et en Amérique du Nord. Toutefois nous avons vu que sa tolérance au froid était plastique : l'utilisation de régimes de température fluctuante permettait de réduire la mortalité liée aux basses températures (**chapitre 2**). Plusieurs études ont montré qu'une autre forme de plasticité, l'acclimation, augmentait grandement la tolérance au froid de cette espèce. Toutefois, les mécanismes physiologiques mis en place lors de l'acclimation sont encore inconnus chez *D. suzukii*. Les mécanismes sous-jacents à l'acquisition de la tolérance au froid chez les insectes sont complexes (voir partie **II.2 de l'introduction**). Afin d'explorer de manière holistique les stratégies sous-jacentes à différentes formes d'acclimation chez *D. suzukii* nous avons utilisé différentes techniques « omiques », nous permettant d'observer les modifications induites par l'acclimation au niveau des voies métaboliques, des phospholipides membranaires, des réserves lipidiques, et de l'expression des gènes de *D. suzukii* (métabolomique : **articles 3 et 4** ; lipidomique : **article 4** ; transcriptomique : **article 5**).



Article 3 : Cold Acclimation Favors Metabolic Stability in *Drosophila suzukii*

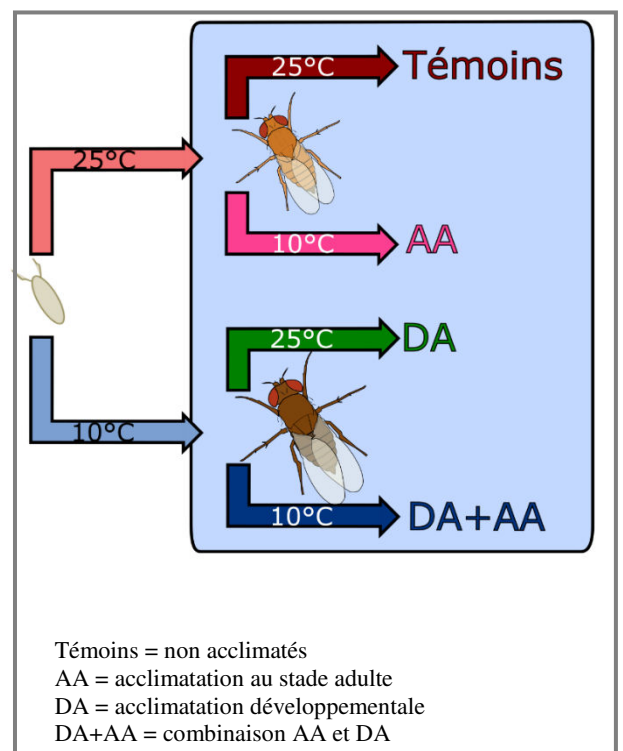
Informations supplémentaires :

Les figures et tableaux supplémentaires mentionnés dans cet article sont disponibles en **annexe 4** et sont également accessibles en suivant le lien ci-dessous :

<https://www.frontiersin.org/articles/10.3389/fphys.2018.01506/full#supplementary-material>

Résumé de l'article en français :

Drosophila suzukii est une espèce intolérante au froid. Pourtant elle est capable de survivre durant l'hiver dans des régions où les températures sont rudes, comme l'Europe du Nord ou l'Amérique du Nord, probablement grâce à une forte plasticité de sa tolérance au froid. Toutefois, les mécanismes sous-jacents à cette plasticité ne sont pas connus chez cette espèce. Dans cette étude, nous avons comparé les effets de différentes formes d'acclimation (durant le développement ou au stade adulte) sur la tolérance au froid de *D. suzukii*. La combinaison de ces deux formes d'acclimation augmentait très fortement la tolérance au froid de *D. suzukii*. Comme c'est le cas chez d'autres espèces de drosophiles, nous nous attendions à observer une accumulation de cryoprotecteurs, ainsi qu'un maintien de l'homéostasie après un stress aigu chez les individus acclimatés. Par la suite, grâce à un profilage quantitatif par GC-MS, nous avons exploré les ajustements métaboliques chez quatre phénotypes de *D. suzukii* : témoins (non acclimatés) ; acclimatés durant le développement ; acclimatés au stade adulte ; acclimatés durant le développement et au stade adulte. Nous avons également réalisé un suivi temporel de ces quatre profils métaboliques afin d'évaluer leur équilibre métabolique en réponse à un stress aigu et pendant la phase de récupération. Les quatre régimes d'acclimation ont produit quatre métabotypes distincts. Les drosophiles acclimatées à la fois durant leur développement et au stade adulte étaient caractérisées par une accumulation de cryoprotecteurs (carbohydrates et acides aminés), mais de faible ampleur (de l'ordre du nmol/mg). Après le stress froid, les drosophiles non acclimatées montraient une perte d'homéostasie, symptomatique des dégâts provoqués par le froid, corrélée avec une augmentation stochastique de la concentration d'acides aminés libres. En revanche, les drosophiles les plus tolérantes au froid montraient un maintien de l'homéostasie métabolique après le stress. Ces résultats indiquent que l'acquisition de la tolérance au froid chez *D. suzukii* est liée à des mécanismes physiologiques communs aux autres espèces du genre *Drosophila* : augmentations modérées des concentrations de cryoprotecteurs couplé à une stabilité métabolique. De plus, nos résultats confirment que les différentes formes d'acclimation testées étaient caractérisées par des mécanismes physiologiques distincts.





Cold Acclimation Favors Metabolic Stability in *Drosophila suzukii*

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The invasive fruit fly pest, *Drosophila suzukii*, is a chill susceptible species, yet it is capable of overwintering in rather cold climates, such as North America and North Europe, probably thanks to a high cold tolerance plasticity. Little is known about the mechanisms underlying cold tolerance acquisition in *D. suzukii*. In this study, we compared the effect of different forms of cold acclimation (at juvenile or at adult stage) on subsequent cold tolerance. Combining developmental and adult cold acclimation resulted in a particularly high expression of cold tolerance. As found in other species, we expected that cold-acclimated flies would accumulate cryoprotectants and would be able to maintain metabolic homeostasis following cold stress. We used quantitative target GC-MS profiling to explore metabolic changes in four different phenotypes: control, cold acclimated during development or at adult stage or during both phases. We also performed a time-series GC-MS analysis to monitor metabolic homeostasis status during stress and recovery. The different thermal treatments resulted in highly distinct metabolic phenotypes. Flies submitted to both developmental and adult acclimation were characterized by accumulation of cryoprotectants (carbohydrates and amino acids), although concentrations changes remained of low magnitude. After cold shock, non-acclimated chill-susceptible phenotype displayed a symptomatic loss of metabolic homeostasis, correlated with erratic changes in the amino acids pool. On the other hand, the most cold-tolerant phenotype was able to maintain metabolic homeostasis after cold stress. These results indicate that cold tolerance acquisition of *D. suzukii* depends on physiological strategies similar to other drosophilids: moderate changes in cryoprotective substances and metabolic robustness. In addition, the results add to the body of evidence supporting that mechanisms underlying the different forms of acclimation are distinct.

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INTRODUCTION

Extreme temperatures often negatively affect survival of ectothermic animals as well as their biological functions such as reproduction, respiration, digestion, or excretion (Chown and Nicolson, 2004; Angilletta, 2009). In order to reduce the negative effects of temperature on their performances, ectotherms are capable of modulating thermal tolerance during their lifetime using a range of physiological adjustments that take place after pre-exposure to sub-lethal temperatures, a phenomenon referred to as thermal acclimation (Angilletta, 2009; Colinet and Hoffmann, 2012). The degree of tolerance acquisition directly depends on the thermal history of individuals, more

particularly on the timing and length of the pre-exposure (Chown and Nicolson, 2004). The capacity to plastically deal with thermal stress is also believed to be a key factor in the success of exotic invasive species (Davidson et al., 2011; Renault et al., 2018).

Developmental plasticity can irreversibly alter some phenotypic traits, such as morphology (Piersma and Drent, 2003). For example, insects developing at low temperature are characterized by a larger body size and darker cuticle pigmentation of adults that remains throughout their whole life (Gibert et al., 2000, 2007). However, physiological adjustments occurring during development, like those related to acquired cold tolerance, are not necessarily everlasting (Piersma and Drent, 2003). For instance, cold tolerance acquired during development is readily adjusted to the prevailing conditions during adult acclimation without a detectable developmental constraint (Slotsbo et al., 2016). The different forms of acclimation probably lie along a continuum of shared common mechanisms; however, several lines of evidence suggest that physiological underpinnings of each acclimation form show some specificity (Colinet and Hoffmann, 2012; Teets and Denlinger, 2013; Gerken et al., 2015).

Stressful low temperatures compromise cells' integrity by altering cytoskeleton structures and membranes' functions (Cottam et al., 2006; Lee et al., 2006; Denlinger and Lee, 2010; Des Marteaux et al., 2017). Cold stress also induces central nervous system shutdown and loss of ions and water homeostasis that result in coma and neuromuscular impairments (Košťál et al., 2004; MacMillan and Sinclair, 2011; Andersen et al., 2016). Alteration of metabolic homeostasis is another symptom of cold stress, likely resulting from downstream consequences such as loss of function of membranes and enzymes (Overgaard et al., 2007; Teets et al., 2012; Williams et al., 2014, 2016; Colinet et al., 2016; Košťál et al., 2016b; Colinet and Renault, 2018). Thermal acclimation likely depends on many concomitant physiological adjustments such as changes in membrane fluidity (e.g., Overgaard et al., 2005; Lee et al., 2006; Košťál et al., 2011a; Williams et al., 2014), preservation of membrane potential and ion balance (Andersen et al., 2016; Overgaard and MacMillan, 2016), maintenance of metabolic homeostasis (e.g., Malmendal et al., 2006; Colinet et al., 2012; Teets et al., 2012), altered expression of heat shock proteins (Colinet and Hoffmann, 2012), and accumulation of substances with cryoprotective functions, such as sugars, polyols and amino acids (Košťál et al., 2011a; Vesala et al., 2012; Foray et al., 2013; MacMillan et al., 2016). Cryoprotective solutes can have beneficial effects at high concentration, by decreasing hemolymph freezing temperature (colligative effect) (Zachariassen, 1985; Storey and Storey, 1991), but also at low concentration, by stabilizing membranes and protein structures (Carpenter and Crowe, 1988; Crowe et al., 1988; Yancey, 2005; Cacula and Hinch, 2006).

The spotted wing drosophila, *Drosophila suzukii*, is an invasive species that is now spread in West and East Europe (Calabria et al., 2012; Lavrinienko et al., 2017) as well as in North and South America (Hauser, 2011; Lavagnino et al., 2018; see also Asplen et al., 2015 for a review). Contrary to other drosophilids, *D. suzukii* females lay eggs in mature fruits. Larvae consume these fruits, causing important damages and economic losses to a wide range of fruit crops (Goodhue et al., 2011; Walsh et al.,

2011). This fly is highly polyphagous and is therefore able to develop on a wide range of wild fruits in addition to those that are cultivated (Poyet et al., 2015; Kenis et al., 2016). This fly is chill susceptible, succumbing to temperatures well above 0°C (Kimura, 2004; Dalton et al., 2011; Ryan et al., 2016; Enriquez and Colinet, 2017). Yet, *D. suzukii* is capable of overwintering in rather cold climates, such as in Northern America and Europe, probably by using various strategies such as migration to favorable microhabitats (Zerulla et al., 2015; Rossi-Stacconi et al., 2016; Tonina et al., 2016; Thistlewood et al., 2018) and/or high cold tolerance plasticity. These strategies allow maintenance of the populations in invaded areas, even if the number of adults is drastically reduced during winter (Mazzetto et al., 2015; Arnó et al., 2016; Wang et al., 2016).

Drosophila suzukii is capable of enhancing cold tolerance via a range of acclimation responses (Hamby et al., 2016). Jakobs et al. (2015) found that exposure at 0°C for 1 h did not trigger a rapid cold hardening response in *D. suzukii*. Conversely, certain lines or populations of *D. suzukii* actually exhibit a typical rapid cold hardening response. Toxopeus et al. (2016) showed that flies acclimated during development could show a rapid cold hardening response after 1 or 2 h at 0°C. Everman et al. (2018) found a similar rapid cold-hardening response in non-acclimated flies exposed at 4°C for 2 h. This fly is also capable of acquiring cold tolerance via acclimation at adult stage (Jakobs et al., 2015; Wallingford and Loeb, 2016), or via developmental acclimation (Toxopeus et al., 2016; Wallingford and Loeb, 2016). In *D. suzukii*, developmental acclimation at temperatures below 12°C (combined or not with short photoperiod) results in a phenotype showing increased body size, dark pigmentation, reproductive arrest, and enhanced cold tolerance; this phenotype, referred to as “winter morph”, is supposed to be the overwintering form of *D. suzukii* (Stephens et al., 2015; Shearer et al., 2016; Toxopeus et al., 2016; Wallingford and Loeb, 2016; Everman et al., 2018). Effect of the different forms of acclimation on subsequent cold tolerance has been rather well described in *D. suzukii*, but surprisingly, the mechanisms underlying cold tolerance acquisition through acclimation in this species are still poorly understood. In order to better appreciate and predict overwintering strategies of *D. suzukii*, knowledge about its thermal biology, and particularly cold stress physiology, is urgently needed (Asplen et al., 2015; Hamby et al., 2016).

A recent transcriptomic study suggested that cold tolerance of winter morphs is associated with an upregulation of genes involved in carbohydrates' metabolism (Shearer et al., 2016). However, it is still not known whether cold-hardy *D. suzukii* flies use any specific cryoprotective arsenal. In this study we proposed the first characterization of metabolic adaptations linked to cold acclimation in *D. suzukii*. First, we aimed at assessing the impact of different forms of acclimation on cold tolerance in this species. To do so, we subjected flies to developmental acclimation, adult acclimation or a combination of both acclimation forms, and then assessed subsequent cold tolerance of adults. We expected that (i) each cold acclimation form would promote cold tolerance, and that (ii) combining cold acclimation during both development and

adult stage would further promote cold tolerance. Second, we intended to explore underlying mechanisms of cold acclimation in *D. suzukii*. Specifically, we performed quantitative target gas chromatography–mass spectrometry (GC-MS) profiling to explore metabolic changes, including accumulation of cryoprotectants, in four different phenotypes resulting from different thermal treatments. We also performed a time-series GC-MS analysis to monitor metabolic homeostasis status during cold stress and recovery. We expected that, as other *Drosophila* species (Overgaard et al., 2007; Košťál et al., 2011a; Colinet et al., 2012; Vesala et al., 2012; MacMillan et al., 2016), cold acclimated *D. suzukii* would be characterized by increased concentrations of some cryoprotectants (sugars, polyols, or amino acids) and would be able to maintain metabolic homeostasis after cold shock contrary to chill susceptible phenotypes.

MATERIALS AND METHODS

Flies Rearing and Thermal Treatments

In this work, we used a wild-type *D. suzukii* population coming from the Vigalzano station of the Edmund Mach Foundation (Italy; 46.042574N, 11.135245E). This line was established in 2011 in Italy, and was sent to our laboratory (Rennes, France) in 2016. For the experimentations, *D. suzukii* was reared in glass bottle (100 mL) supplied with a carrot-based food (agar: 15 g, sucrose: 50 g, carrot powder: 50 g, brewer yeast: 30 g, cornmeal: 20 g, Nipagin: 8 mL, tap water: 1 L). Flies were maintained at 25°C, 60% RH, 12 L:12 D into incubators (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd., Gunma, Japan). At least 12 bottles (each containing 100–300 flies) were used to continuously maintain the line at Rennes, and flies from different bottles were crossed every generation to limit inbreeding.

To generate flies for the experiments, groups of approximately 100 mature (7 days old) males and females were placed in 100 mL bottles containing food medium, and females were allowed to lay eggs during 48 h at 25°C. Flies were then removed and bottles with eggs were randomly placed under the different thermal treatments to continue development (Figure 1). Bottles containing eggs were directly placed either at 25°C (12 L:12 D) [no acclimation] or cold acclimated at 10°C (10 L:14 D) [developmental acclimation] (Figure 1). Flies took about 10 and 60 days to start emergence at 25 and 10°C, respectively. At emergence, adults from both developmental conditions were directly placed either at 25°C (12 L:12 D) [control, no acclimation] or cold acclimated at 10°C (10 L:14 D) [adult acclimation] with fresh food for seven consecutive days. Flies were transferred into new bottles containing fresh food every 2 days. Thereby, four different phenotypes that experienced four different thermal treatments were generated: non-acclimated control, adult acclimation, developmental acclimation and combined developmental and adult acclimation hereafter referred to as combined acclimation (Figure 1). All experiments were conducted on males, which were separated from females visually (with an aspirator) without CO₂ to avoid stress due to anesthesia (Colinet and Renault, 2012).

Recovery From Acute Cold Stress

Males randomly taken from each treatment group were distributed in 10 replicates of 10 individuals and were subjected to an acute cold stress using 10 glass vials that were directly immersed in a glycol solution cooled by a cryostat (Cryostat Lauda ECO RE 630) at –5°C for 100 min. In a previous experiment, this combination of temperature and duration induced 40% survival in non-acclimated *D. suzukii* (Enriquez and Colinet, 2017). After exposure, flies were directly transferred in 40 mL food vials and allowed to recover at 25°C (12 L: 12 D). Survival was assessed by counting the number of dead and living individuals in each vial 4, 24, and 48 h after the acute cold stress.

Critical Thermal Minimum (Ct_{min})

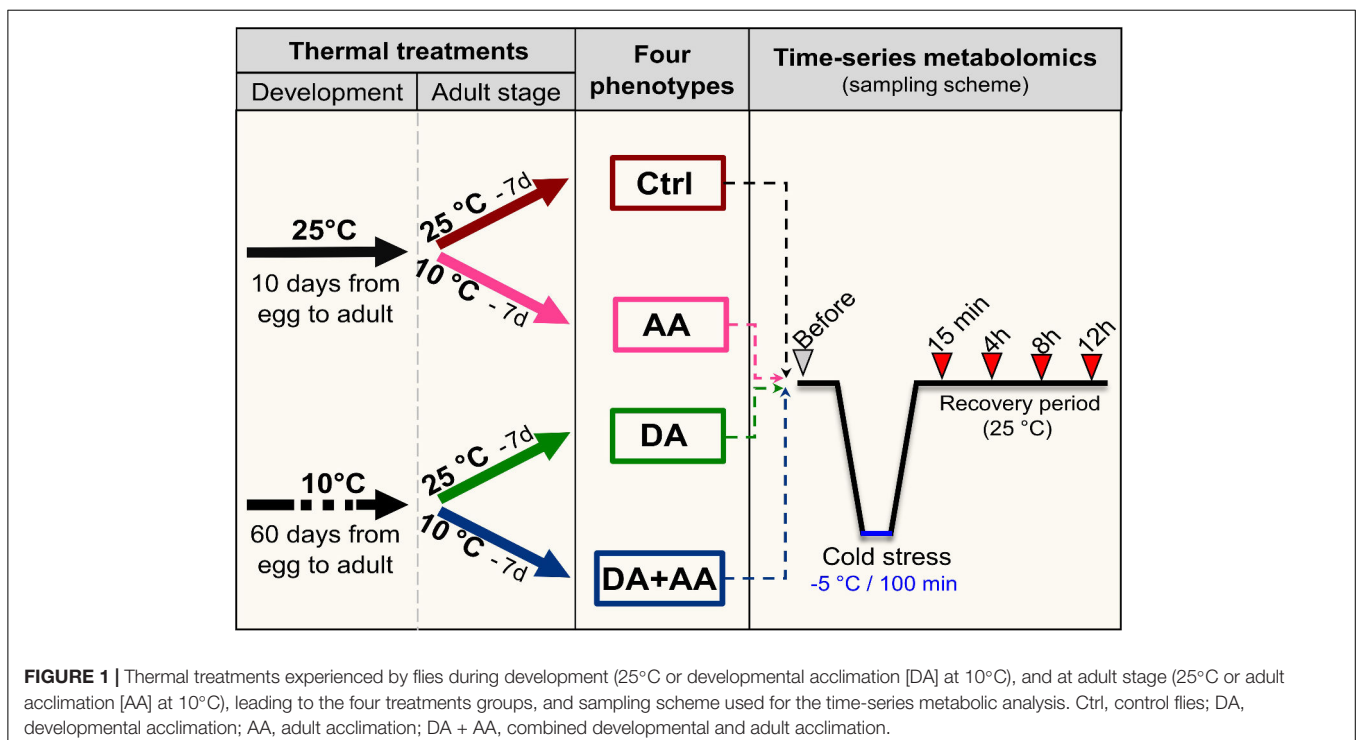
To measure Ct_{min}, we used a glass knockdown column that consisted of a vertical jacketed glass column (52 × 4.7 cm) containing several cleats to help flies not fall out the column while still awake. In order to regulate the temperature, the column was connected to a cryostat (Cryostat Lauda ECO RE 630), and temperature was checked into the column using a thermocouple K connected to a Comark Tempscan C8600 scanning thermometer (Comark Instruments, Norwich, United Kingdom). Thermocouple was positioned at the column center, at mid height through a tiny insulated hole. For each condition, approximately 60 flies were introduced at the top of the column. Flies were allowed to equilibrate in the device for few minutes, and then the temperature was decreased to –5°C at 0.5°C/min. For each individual fly falling out of the column, the Ct_{min} (°C) was recorded.

Chill Coma Recovery Time

Chill coma recovery time (CCRT) is defined as the resurgence time of motor activity after a cold knockdown (David et al., 1998). In order to induce coma, 40 males of each experimental condition were subjected to 0°C for 12 h, using a vial placed directly in a cooled-incubator. We choose this temperature and duration because in a previous experiment we showed that 12 h at 0°C induced a relatively small mortality level in non-acclimated males (Enriquez and Colinet, 2017). Upon removal, adults were positioned supinely on a table in a thermally controlled room (25 ± 1°C), using a fine paintbrush, and the time to regain the ability to stand (i.e., CCRT) was monitored individually. Experimentation lasted 60 min, and flies that did not recover by that time were marked as not recovered (censored).

GC-MS Metabolic Profiling

To assess the effect of the different thermal treatments on metabolic profiles, we sampled flies at the end of each thermal treatment period (i.e., before the stress exposure) (Figure 1). Then, males randomly taken from each treatment group were cold-stressed at –5°C for 100 min (as explained above), and these stressed flies were then sampled during the recovery period after 15 min, 4, 8, and 12 h (Figure 1). For each time-point, seven replicates, each consisting of a pool of 10–12 living flies, were snap-frozen in liquid N₂ and stored at –80°C. Fresh mass of



each sample was measured prior to metabolites' extraction using a microbalance (Mettler Toledo UMX2, accuracy 0.001 mg).

Samples were homogenized using a bead-beating device (Retsch MM301, Retsch GmbH, Haan, Germany) at 20 beats per second during 90 s in 600 μL of a cold solution of methanol-chloroform (2:1) with a tungsten grinding ball. Samples were then kept at -20°C for 30 min. A volume of 400 μL of ultrapure water was added to each tube, and samples were vortexed. After centrifugation at 4000 g for 10 min at 4°C , 120 μL aliquots of the upper aqueous phase containing polar metabolites were transferred to microtubes and vacuum-dried (SpeedVac Concentrator, miVac, Genevac Ltd., Ipswich, England). The dried polar phase aliquots were resuspended in 30 μL of 20 mg mL^{-1} methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, United States) in pyridine, and incubated under orbital shaking at 40°C for 60 min. Afterward, 30 μL of N_2O -Bis(trimethylsilyl)trifluoroacetamide (BSTFA; Sigma-Aldrich) was added, and derivatization was performed at 40°C for 60 min under continuous agitation.

Gas chromatography/mass spectrometry was used to quantify primary metabolites (non-structural carbohydrates, polyols, amino and organic acids), as described in Colinet et al. (2016). Briefly, GC-MS consisted of a CTC CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland), a Trace GC Ultra chromatograph, and a Trace DSQII quadrupole mass spectrometer (Thermo Fischer Scientific Inc., Waltham, MA, United States). The autosampler enabled online derivatization and standardization of the preparation process: each sample was automatically prepared during GC analysis of the preceding sample, ensuring the highest

possible throughput of the system, and resulting in equal derivatization duration for each compound prior to injection. For each sample, 1 μL was injected in the oven using the split mode (25:1; temperature of the injector: 250°C). The oven temperature ranged from 70 to 147°C at 9°C min^{-1} , from 147 to 158°C at $0.5^\circ\text{C min}^{-1}$, from 158 to 310°C at 5°C min^{-1} and held at 320°C for 4 min. Helium was the gas carrier (1 mL min^{-1}) and MS detection was achieved using electron impact. All samples were run under the SIM mode (electron energy: -70 eV), thereby, we only screened for the 59 pure reference compounds included in our spectral database. GC-MS peaks were accurately annotated using both mass spectra (two specific ions), and retention index specific to each compound. Calibration curve samples for 59 pure reference compounds at 10, 20, 50, 100, 200, 500, 700, and 1000 μM concentrations were run. Chromatograms were deconvoluted using XCalibur 2.0.7, and metabolite levels were quantified using the quadratic calibration curves for each reference compound and concentration. Concentrations were then normalized with the sample weight.

Statistical Analyses

All analyses (except survival analyses of CCRT curves) were conducted using R (version 3.4.3; R Core Team, 2016). We modeled survival of flies exposed at -5°C by specifying a generalized linear mixed-effects model (GLMM) with logit link function for proportions outcome (i.e., number of dead/alive flies per vial). The response variable was dependent on the developmental temperature (10 vs. 25°C), the temperature at adult stage (10 vs. 25°C), the time to survival measurement and the interaction among terms. Vial number was considered as

random effect. We analyzed the effect of each variable through an analysis of deviance (“Anova” function in “car” package, Fox and Weisberg, 2011). Differences among treatment groups were analyzed by comparing least-squares means using the “emmeans” package (Russell, 2018).

$C_{t_{min}}$ data were analyzed using a two-way ANOVA, dependent on developmental (10 vs. 25°C) and adult temperatures (10 vs. 25°C), and the interaction between these two factors. Then, a Tukey HSD comparison was used to identify differences between the interaction of these two factors.

CCRT data were analyzed using survival analyses with the software GraphPad Prism5. We compared recovery curves using Gehan-Breslow-Wilcoxon Tests. We adjusted the alpha level of significance thanks to Bonferroni correction ($\alpha = 0.008$).

Concentrations of each metabolite after the four thermal treatments have been checked for normality: for some metabolites, normality was not respected, but total number of individuals and sample size were sufficient to use one-way ANOVAs (Blanca et al., 2017). Tukey HSD comparisons were then used to identify differences between thermal treatments. Metabolite concentrations were also pooled in groups corresponding to their respective biochemical families (i.e., amino acids, carbohydrates, polyols, etc.). Similarly, concentrations of biochemical families were analyzed using one-way ANOVAs followed by Tukey tests.

If, as speculated, cold acclimated phenotypes are characterized by a post-stress metabolic inertia, while non-acclimated counterparts loose metabolic homeostasis, then, different temporal patterns should be observed during recovery period among phenotypic groups. This should result in significant time \times treatment interactions, at both univariate and multivariate levels. Temporal changes in the concentrations of metabolites and biochemical families were analyzed using GLMs with Gaussian link function, and the effect of the interaction between the thermal treatments and time after the cold stress were analyzed through an analysis of deviance (“Anova” function in “car” package, Fox and Weisberg, 2011).

Data resulting from quantitative GC-MS analysis were also analyzed using several multivariate tests. The metabolites' compositions resulting from each phenotype (i.e., the metabolite) were compared using between-class principal component analysis (PCA) (Dolédéc and Chessel, 1991) in the “ade4” package in R (Dray and Dufour, 2007). A first PCA was performed using metabolic datasets of the four treatments before stress exposure (see **Figure 1**), in order to identify main patterns and clustering resulting from the different thermal conditions. A second PCA was performed on the time-series datasets of the four phenotypes in order to monitor metabolic homeostasis status during recovery period (see **Figure 1**). Monte Carlo tests were performed to examine the significance of the difference among the classes (based on 1000 simulations). To identify the variables (i.e., metabolites) contributing the most to the PCA structure separation, the correlations to the principal components (PCs) were extracted and ranked. Data were scaled and mean-centered prior to the PCAs. In addition to the classical PCA, an ASCA was performed to further analyze the time-series datasets. The ASCA (ANOVA-Simultaneous

Component Analysis) is a multivariate approach ideal for the analysis of time-series metabolomic studies (Smilde et al., 2005). ASCA applies a PCA to the estimated parameters for each source of variation of the model and tests the main effects (i.e., thermal treatment and time) as well as the interaction. If acclimated phenotypes have a distinct and more efficient homeostatic response than control chill-susceptible group, then different temporal metabolic trajectories should be observed, and this should result in a significant interaction effect in the ASCA. The ASCA was performed using the temporal analysis module of MetaboAnalyst 4.0 (Chong et al., 2018). This pipeline tests the main and the interaction effects via permutation tests.

RESULTS

Cold Tolerance

Survival to Acute Cold Stress

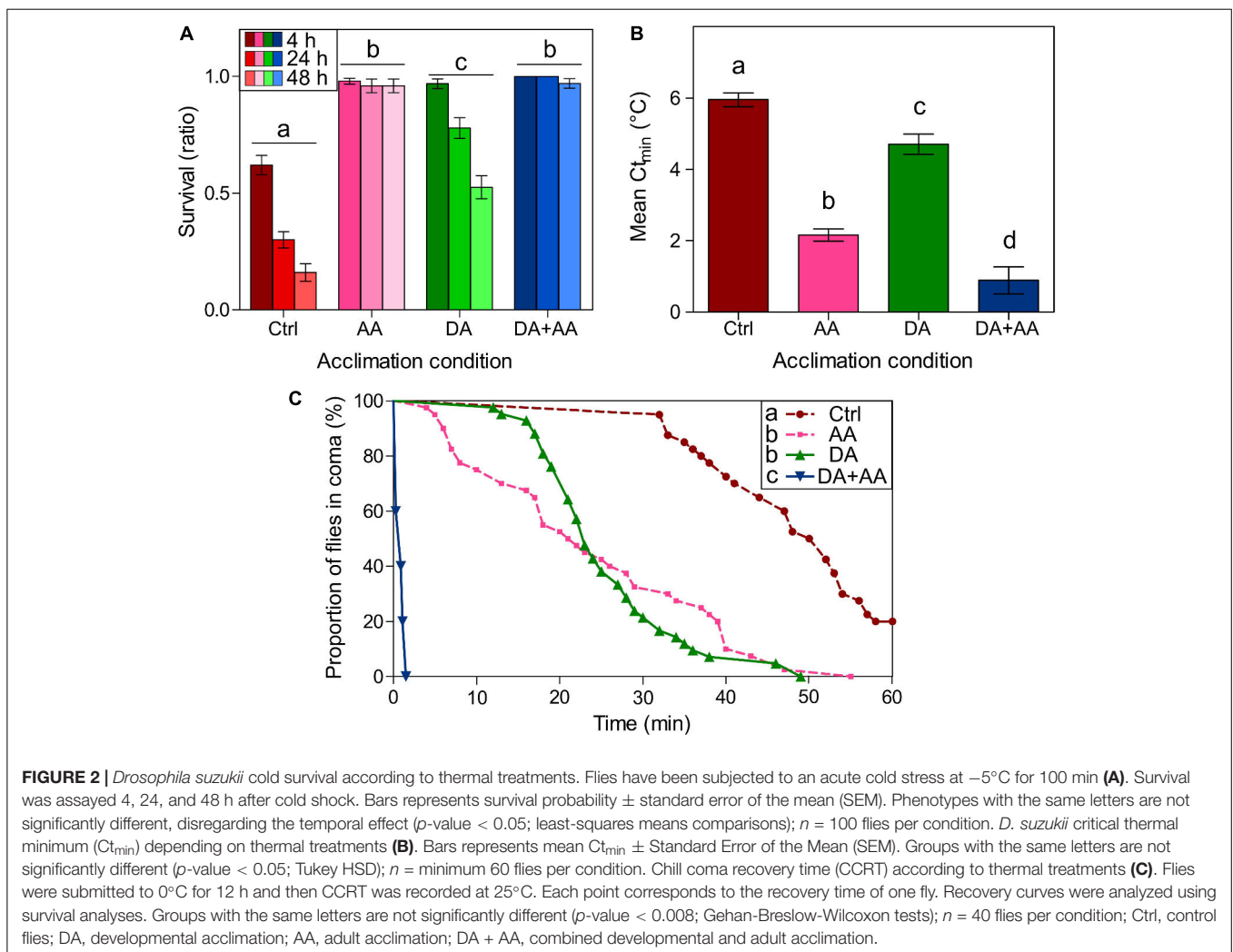
Figure 2A illustrates survival probability of the flies 4, 24, and 48 h after a cold shock (-5°C) in the four phenotypes. Flies from combined acclimation were the only showing 100% survival, at 4 and 24 h post stress. GLMM model showed an effect of developmental conditions and rearing temperature at adult stage on cold survival ($\chi^2 = 40.41$, $df = 1$; p -value < 0.001 ; $\chi^2 = 107.36$, $df = 1$, p -value < 0.001 , respectively). Survival decreased with increased time of assessment after cold exposure, particularly in control and developmental acclimation ($\chi^2 = 84.98$, $df = 1$, p -value < 0.001). Outcomes from least-square means comparisons are available on **Figure 2A**: globally, flies from combined acclimation and adult acclimation showed the highest survival rates (97 and 96% after 48 h, respectively), followed by flies submitted to developmental acclimation and control group that showed the lowest survival rate (52 and 16% after 48 h, respectively).

$C_{t_{min}}$

$C_{t_{min}}$ values of the four phenotypes are illustrated in **Figure 2B**. $C_{t_{min}}$ was affected by developmental conditions and temperature at adult stage [$F_{(1,215)} = 26.56$, p -value < 0.001 ; $F_{(1,215)} = 230.20$, p -value < 0.001 , respectively]. Outcomes from Tukey HSD comparisons are available on **Figure 2B**: $C_{t_{min}}$ values of the four phenotypes were all significantly different, flies from combined acclimation had the lowest $C_{t_{min}}$ (0.89°C), followed by flies from adult acclimation (2.16°C), developmental acclimation (4.70°C), and finally control flies which had the highest $C_{t_{min}}$ (5.95°C).

CCRT

CCRT curves are displayed in **Figure 2C**. In flies from combined acclimation group, it took no more than two minutes for the flies to recover, in the other groups recovery took much longer. Consequently, flies from combined acclimation were the very first to recover, followed by flies submitted to adult acclimation and developmental acclimation, and finally controls flies took the longer time to recover: after one hour of recovery, 20% of



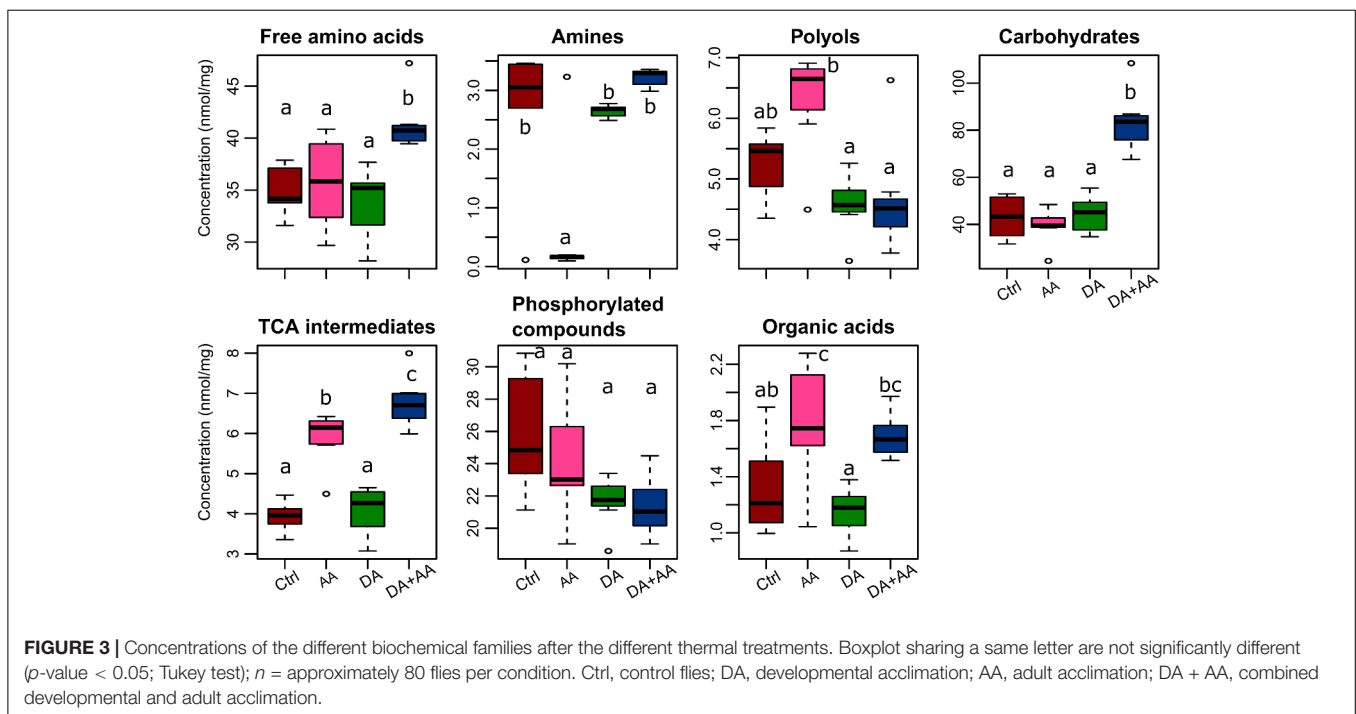
the control flies were still in coma. Outcomes from the Gehan-Breslow-Wilcoxon tests are available on **Figure 2C**.

Metabolic Characterization of Acclimation

Metabolite Concentrations After Acclimation

Among the 59 metabolites included in our spectral library, 45 were identified and quantified (i.e., absolute quantification). The list of identified metabolites, their respective abbreviations and their biochemical families are presented in **Supplementary Table S1**. **Figure 3** presents the variation patterns by biochemical classes at the end of thermal treatments. Outcomes resulting from Tukey tests on the different biochemical families according to thermal treatments are available on **Figure 3**. Except for phosphorylated compounds, mean concentrations of all biochemical families varied according to thermal conditions experienced during development and as adult (**Figure 3**). The most striking changes were observed in flies submitted to combined acclimation that were characterized by significant increases in free amino acids, carbohydrates and tricarboxylic

acid (TCA) intermediates in comparison to control flies (1.15-, 2-, and 6-fold change, respectively; **Figure 3**). Flies from adult acclimation displayed a significant decrease in amines and increased levels of TCA intermediates relative to control (0.03- and 1.5-fold change, respectively; **Figure 3**). These global patterns were driven by concentration changes in individual's metabolites that are all available in the panel **Supplementary Figures S1–S7**. Flies from the combined acclimation showed high amounts of several free amino acids (Ala, Glu, Ile, Leu, Phe, Ser, Val; **Supplementary Figure S1**), carbohydrates (Fru, Mal, Man, Suc, Tre; **Supplementary Figure S4**), TCA intermediates (citrate, malate, succinate; **Supplementary Figure S5**), and the other compounds GABA and GDL (**Supplementary Figure S7**). In this phenotypic group, a decrease of F6P and G6P was observed compared to control (**Supplementary Figure S6**). In comparison to control group, flies submitted to adult acclimation were characterized by an accumulation of several polyols (glycerol, mannitol, and xylitol; **Supplementary Figure S3**) and two TCA intermediates (malate, citrate; **Supplementary Figure S5**) and a decrease in spermine, F6P and G6P (**Supplementary Figures S2, S6**). Patterns of flies submitted to developmental



acclimation were relatively similar to those of control flies, only two amino acids had increased amounts (Ser and Thr), while three compounds had decreased concentrations (Ala, Cit, and Phe) (Supplementary Figure S1).

Multivariate Analysis on Metatypes After Acclimation

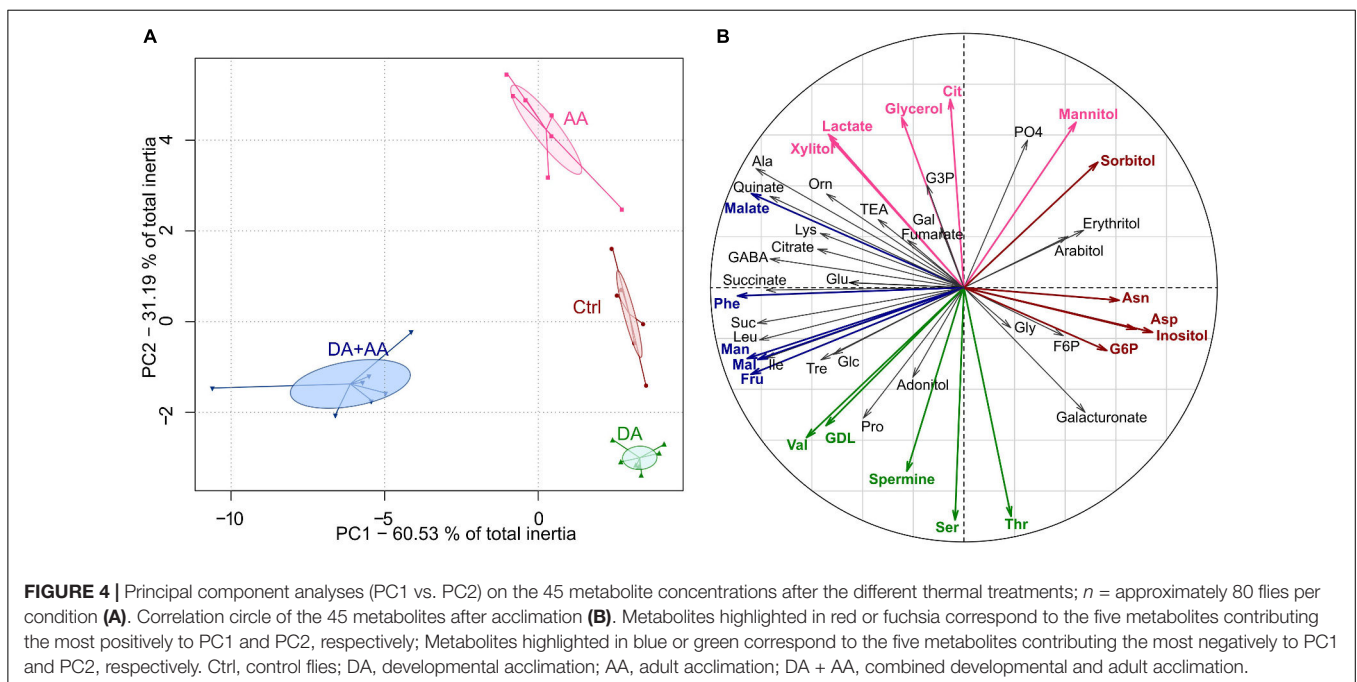
Global metabolic changes according to thermal treatments were also characterized using a PCA, and the ordination of classes within the first plane is presented in Figure 4A. This multivariate analysis revealed a clear-cut non-overlapping separation among the four thermal treatments (Figure 4A). The metatype reflecting combined acclimation was the most divergent and was opposed to the other metatypes on the first axis of the PCA (PC1, 60.53% of total inertia). The three other metatypes separated along the second axis (PC2, 31.19% of total inertia). Thus, PC1 and PC2 supported 91.72% of total inertia. The Monte-Carlo randomizations corroborated the significance of the differences among classes (i.e., treatments; observed p -value < 0.001). The projection of the variables of the PCA (i.e., metabolites) on the correlation circle are shown in panel Figure 4B and mirror concentration changes of individual metabolites (Supplementary Figures S1–S7).

Temporal Patterns of Metabolic Profiles During the Recovery From Acute Cold Stress

Variation of Metatypes During Recovery From Acute Cold Stress

Metabolic compositions of the four thermal treatments were monitored before the stress, and during the post-stress recovery

period. Figure 5 displays temporal changes of concentrations of metabolites' biochemical families. Univariate statistics from GLMs describing the effects of recovery time, thermal treatments and their interaction are available in Table 1. Interactions (time \times treatment) were found for all the biochemical families (Figure 5 and Table 1) suggesting divergent homeostatic trajectories among thermal treatments. This was reflected for instance in the pool of free amino acids or polyols that remained relatively stable in flies submitted to combined acclimation, whereas it increased in other phenotypes (Figure 5). Flies from combined acclimation were the only to show temporal decrease in global concentration of carbohydrates (0.75-fold change) and TCA intermediates dropped from 7 to 5 nmol/mg after 12 h of recovery (Figure 5). These global temporal patterns were driven by concentration changes in individual's metabolites that are all available in the panel Supplementary Figures S8–S14, together with the statistics showing the significance of treatment \times time interactions in Supplementary Table S2. Most of the metabolites showed significant treatment \times time interaction (Supplementary Table S2). During the recovery from cold stress, control flies were characterized by increased concentrations in most metabolites, including free amino acids (Cit, Glu, Ile, Leu, Lys, Orn, Phe, Pro, Thr, and Val; Supplementary Figure S8), polyols (adonitol, erythritol, glycerol, sorbitol, and xylitol; Supplementary Figure S10), TEA, one TCA intermediate (citrate), PO4, galacturonate and GDL (Supplementary Figures S9, S12–S14). Flies from developmental acclimation also showed temporal increase in the concentrations of free amino acids (e.g., Ala, Glu, Ile, Leu, Phe, Pro, Thr, and Val; Supplementary Figure S8). Most of the quantified polyols accumulated at 4 or 8 h post-stress and returned to their initial concentrations after 12 h of recovery (Supplementary Figure S10). Furthermore, these

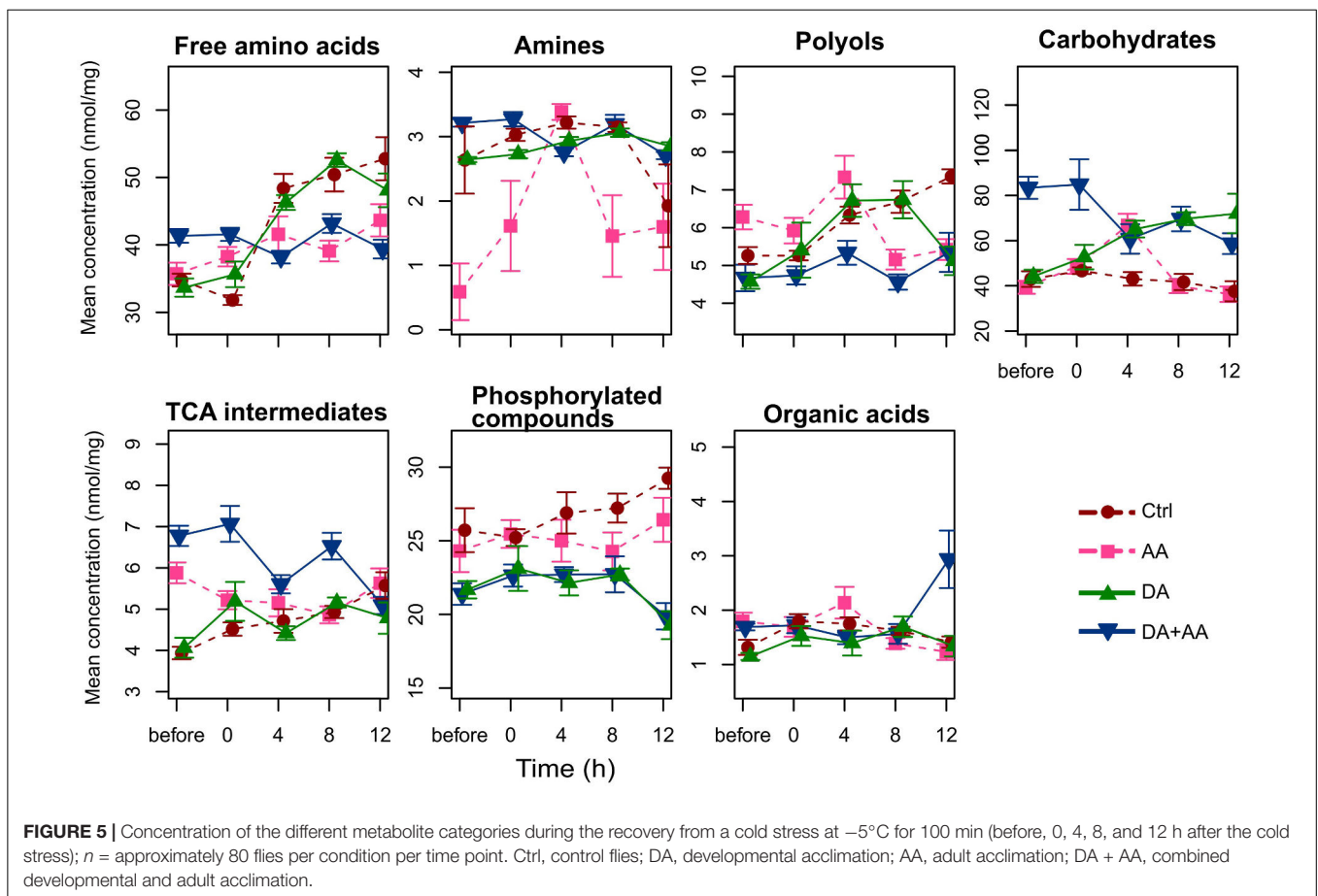


flies showed a temporal accumulation of several carbohydrates (Fru, Man, and Tre; **Supplementary Figure S11**) and GDL (**Supplementary Figure S14**), and a light decrease in the level of phosphorylated compounds (F6P and G6P; **Supplementary Figure S13**). Flies from the adult acclimation showed temporal variations in the level of several free amino acids (decrease of Ala, increase of Asp, Ile, Leu, Pro, Ser, and Val; **Supplementary Figure S8**), increased amounts of amines (TEA and spermine; **Supplementary Figure S9**), succinate and a decrease of the concentration of malate (**Supplementary Figure S12**). The concentration of several polyols (adonitol, erythritol, mannitol, and xylitol; **Supplementary Figure S10**), carbohydrates (Fru, Glc, Man, and Tre; **Supplementary Figure S11**), organic acids (galacturonate, lactate, and quinate) and GABA and GDL (**Supplementary Figure S14**) showed a similar temporal pattern: the concentration increased 4 h after the cold stress and returned to initial concentration within 8 or 12 h after the stress. Finally, flies submitted to combined acclimation showed a decrease in Ala and Phe concentration during recovery from cold stress (**Supplementary Figure S8**). Other free amino acids (Gly, Ile, Leu, Ser, and Val; **Supplementary Figure S8**) showed increased levels during the recovery period but returned to initial concentrations after 12 h of recovery. The level of carbohydrates (Fru, Glc, Mal, Man, and Tre; **Supplementary Figure S11**) and TCA intermediates (succinate and malate; **Supplementary Figure S12**) moderately decreased with recovery time, while lactate accumulated after the cold stress (**Supplementary Figure S12**).

PCA on Metabolite Compositions During Recovery From the Acute Cold Stress

A PCA was made on metabolic patterns across the different sampling times (**Figure 6A**). Temporal changes among

metabotypes were mainly reflected along PC1 (29.99% of total inertia), while differences between thermal treatments were associated with PC2 (24.06% of total inertia; PC1 + PC2 = 54.05% of total inertia). Metabotypes for control showed a marked temporal deviation along PC1. Metabotypes for adult and developmental acclimation also showed temporal deviations from initial status (especially after 8 h of recovery), but less pronounced than in the control group. Metabotypes for combined acclimation were again clearly distinct from the other groups on PC2 and showed a temporal deviation of metabolic status after 4 and 8 h of recovery, but after 12 h of recovery the metabolic profiles had returned to initial state on PC1 (**Figure 6A**). These differences among metabotypes were validated by Monte Carlo randomizations (observed p -value < 0.001). The temporal changes of these metabotypes can be observed in **Figure 6B**, which represents the projections of the centroid scores on PC1. From these, it can clearly be discerned that flies from control and developmental acclimation showed the most intense temporal deviations from initial states, while counterparts from combined acclimation showed much moderate deviation, and a returned to the initial metabolic state within 12 h. Flies from adult acclimation showed an intermediate response. Correlation of metabolites with PC1 and PC2 are available in **Figures 6C,D**, respectively. A large number of free amino acids (e.g., Pro, Thr, and Val) and polyols (e.g., erythritol, inositol, and sorbitol) were positively correlated to PC1 (**Figure 6C**), and these changes reflected temporal increase in metabolites' concentrations, mainly in control flies (see **Figure 5** and **Supplementary Figure S2**). Several carbohydrates (e.g., Fru, Man, Suc, and Mal) were positively correlated with PC2 (**Figure 6D**), revealing relative higher amounts of these metabolites in flies from combined acclimation compared to control flies (see also **Figure 5** and **Supplementary Figure S2**).



ASCA on Metabolite Compositions During Recovery From Acute Cold Stress

The results of ASCA are presented in panel **Supplementary Figure S15**. The **Supplementary Figure S15A** shows the major patterns associated with the factor “treatment” (i.e., thermal treatments) of the ASCA. A decreasing trend was detected, with flies from combined acclimation showing positive score. Control flies were at the opposite, with a negative score and flies from developmental and adult acclimation were intermediate (**Supplementary Figure S15A**). This pattern mirrored the PCA results shown in **Figure 6A**. The **Supplementary Figure S15B** shows the major patterns associated with the factor “time”. The scores was initially negative (before the stress) and remained stable after the stress (0 h), then a marked increase occurred at 4 h of recovery (time 4), when the score became positive, after which the scores remained positive at 8 and 12 h of recovery. These results are also consistent with PCA results shown in **Figures 6A,B**, in which the metabolites of flies showing the lowest cold tolerance (control and developmental acclimation mainly) shifted in the same direction on PC1 (toward positive scores) over the time-course of the experiment. Interestingly, the major patterns associated with interaction terms suggested that flies from combined and adult acclimation had rather similar temporal patterns that differed from that of flies from developmental acclimation

and control (**Supplementary Figure S15C**), suggesting a major importance of adult acclimation. Finally, the observed statistics with permutation tests were significant for both main factors (observed p -value < 0.05 for both “treatment” and “time”), confirming the clear differentiation of metabolotypes according to both “treatment” and “time” (**Supplementary Figure S15D**). More interestingly, the permutation test for “treatment \times time” interaction was also significant (observed p -value < 0.05), demonstrating that temporal metabolic trajectories significantly differed according to thermal treatments (**Supplementary Figure S15D**).

DISCUSSION

Impact of Thermal Acclimation on *D. suzukii* Cold Tolerance

In this study, we subjected *D. suzukii* flies to four different thermal treatments to estimate the effect of developmental and adult acclimation or the combination of these two forms of acclimation on cold tolerance of adults. As expected, combining developmental and adult acclimation led to the highest cold tolerance of flies, based on three different measures of cold tolerance (survival, $C_{t_{min}}$, CCRT; **Figure 2**). These results confirm previous observations of Stephens et al. (2015),

Shearer et al. (2016), or Toxopeus et al. (2016), who showed that combination of developmental and adult acclimation leads to a highly cold tolerant phenotype in *D. suzukii*. In *Drosophila melanogaster*, the combination of developmental and adult acclimation also resulted in cumulative effects (Colinet and Hoffmann, 2012; Slotsbo et al., 2016), but this pattern is not a general rule in insects (Terblanche and Chown, 2006). The fact that acclimation benefits cumulated in *D. suzukii* further adds to the widely accepted vision that physiological underpinnings of the different forms of acclimation are somewhat specific (Colinet and Hoffmann, 2012; Teets and Denlinger, 2013; Gerken et al., 2015).

Both developmental and adult acclimations, when applied alone, increased cold tolerance of *D. suzukii*. However, the promoting effect was higher when these two acclimation forms were combined. Developmental and adult acclimations are well known to increase cold tolerance of *D. melanogaster* (Sinclair and Roberts, 2005; Rako and Hoffmann, 2006; Overgaard et al., 2008; Košťál et al., 2011a; Colinet and Hoffmann, 2012) and *D. suzukii* (Jakobs et al., 2015). Here, we found that adult acclimation improved both survival and $C_{t_{min}}$ more intensely than developmental acclimation, though these two acclimation treatments resulted in similar CCRT. It is assumed that the thermal conditions experienced just before a stress mainly determine subsequent cold tolerance (Geister and Fischer, 2007; Fischer et al., 2010; Colinet and Hoffmann, 2012). Thus, the higher effect of adult acclimation on cold tolerance could be due to its “temporal proximity” with the acute cold stress, whereas

seven days at 25°C separated developmental acclimation from the cold shock (Figure 1). It is also possible that flies from developmental acclimation deacclimated during this period.

In this study, light cycles were not strictly identical among the experimental treatments. However, we reasoned that temperature is the main driver of the observed phenotypic changes (cold tolerance). Bauerfeind et al. (2014) reported that stress resistance traits (cold, heat, starvation, and desiccation resistance) are predominantly affected by temperature and not by photoperiod in *D. melanogaster*. Likewise, in *D. suzukii*, delayed reproductive maturity (i.e., the reproductive diapause syndrome) seems temperature-dependent and not regulated by photoperiod (Toxopeus et al., 2016). We therefore assumed that effects observed here on cold tolerance and physiology were primarily due to temperature.

Acclimation Triggers Metabolic Changes in *D. suzukii*

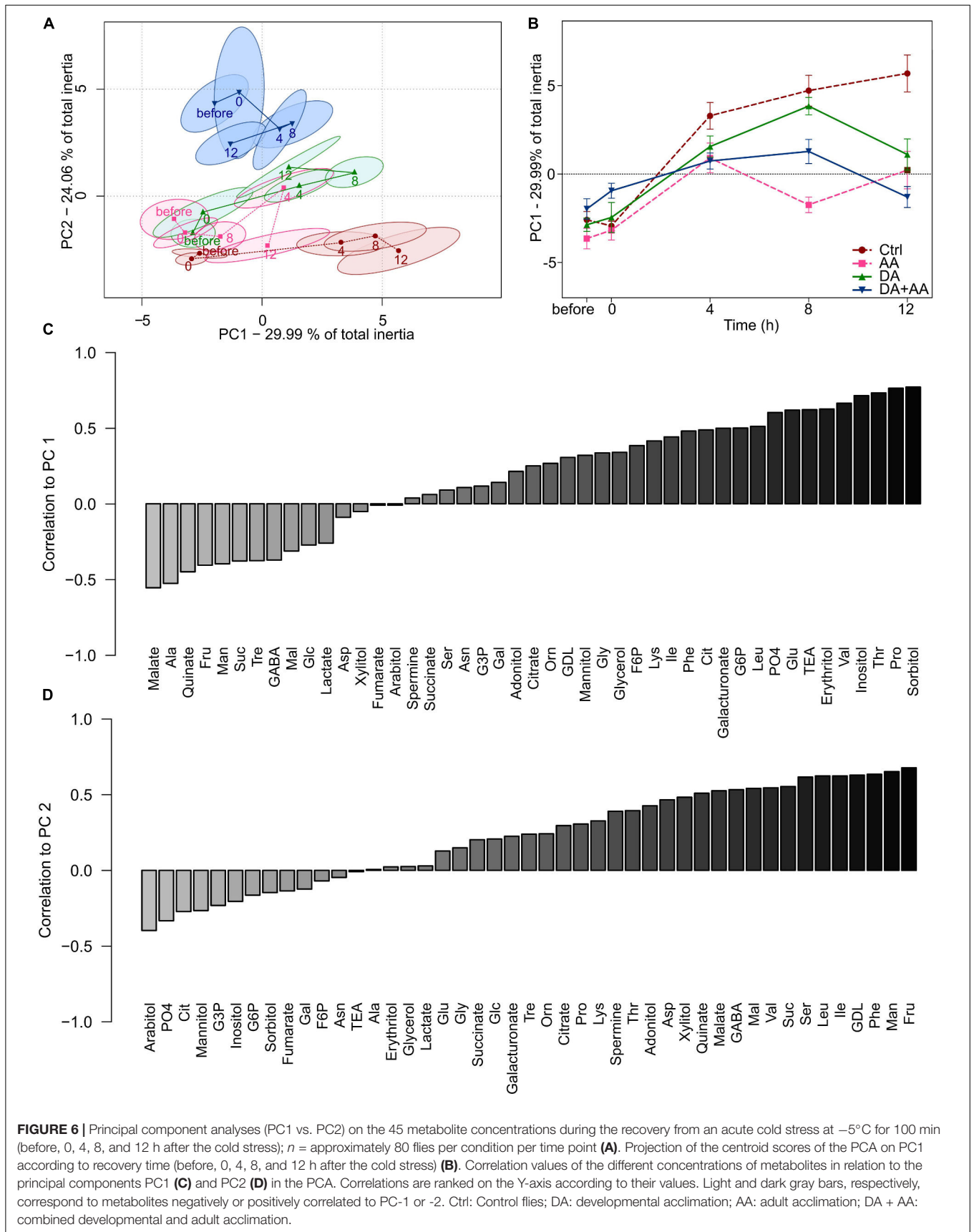
One aim of this study was to identify metabolic changes resulting from different forms of cold acclimation in *D. suzukii*. We expected that the most cold-tolerant phenotypes would be characterized by accumulation of cryoprotectant molecules, such as carbohydrates, polyols or amino acids. GC-MS analysis revealed four non-overlapping metabolotypes, suggesting different metabolic profiles among the four phenotypic groups (Figure 4). Amounts of several carbohydrates, such as Fru, Mal, Man, Suc, and Tre, increased in flies submitted to combined acclimation compare to controls (Supplementary Figure S4). Carbohydrates are known to be involved in cold tolerance of several insect species (Baust and Edwards, 1979; Kimura, 1982; Fields et al., 1998; Zeng et al., 2008; Ditrich and Košťál, 2011). In *D. melanogaster*, cold acclimation and rapid cold hardening increase the level of Fru, Glc, Mal, Suc, and Tre (Overgaard et al., 2007; Košťál et al., 2011a; Colinet et al., 2012). Even if caution should be exercised in designating a function to any upregulated or downregulated metabolite, as flux and pathways are unknown, the increased sugar level after combined acclimation in *D. suzukii* reverberates the results of Shearer et al. (2016). These authors found upregulations of gene clusters involved in the carbohydrates’ metabolism in winter morphs of *D. suzukii*. In this species, the cold-hardy “winter morph” is generated using a combination of developmental and adult acclimation (Stephens et al., 2015; Shearer et al., 2016; Toxopeus et al., 2016; Wallingford and Loeb, 2016). Unlike proper cold tolerant overwintering species that accumulate several hundred mmol L^{-1} of cryoprotectants (Salt, 1961), carbohydrate accumulation in cold acclimated drosophilids is of rather low magnitude, suggesting a non-colligatively contribution to cold hardiness (Košťál et al., 2011a; Colinet et al., 2012), for instance by stabilizing macromolecule structures (Arakawa and Timasheff, 1982; Crowe et al., 1988; Cacula and Hinch, 2006).

In comparison to controls, flies from the adult acclimation had increased concentrations of several polyols (glycerol, mannitol, and xylitol; Supplementary Figure S3). However, as for sugars, the concentrations and magnitude of changes remained too low to consider this pattern as a proper cryoprotective and

TABLE 1 | Outcomes of GLMs on metabolites’ biochemical family concentrations during the recovery from the cold stress.

Biochemical families	Parameter	χ^2	df	p-Value
Free amino acids	Treatment	18.19	3	< 0.001***
	Time	117.59	4	< 0.001***
	Treatment × Time	97.80	12	< 0.001***
Amines	Treatment	45.75	3	< 0.001***
	Time	16.18	4	< 0.01**
	Treatment × Time	34.03	12	< 0.001***
Polyols	Treatment	35.00	3	< 0.001***
	Time	27.58	4	< 0.001***
	Treatment × Time	51.52	12	< 0.001***
Carbohydrates	Treatment	107.05	3	< 0.001***
	Time	7.56	4	0.1
	Treatment × Time	65.17	12	< 0.001***
TCA intermediates	Treatment	92.47	3	< 0.001***
	Time	7.94	4	0.09
	Treatment × Time	64.71	12	< 0.001***
Phosphorylated compounds	Treatment	82.74	3	< 0.001***
	Time	2.24	4	0.69
	Treatment × Time	22.81	12	< 0.05*
Organic acids	Treatment	14.51	3	< 0.01**
	Time	4.17	4	0.38
	Treatment × Time	55.45	12	< 0.001***

Treatment, acclimation treatment; Time, recovery time.



colligative response. Polyols, and especially glycerol, are common cryoprotectants in cold-adapted insects (Sømme, 1982; Storey and Storey, 2005; Denlinger and Lee, 2010). Accumulation of massive concentrations of polyol are linked to freeze-protective functions such as the diminution of the supercooling point (Crosthwaite et al., 2011). On the other hand, at low concentrations, polyols may play other protective functions, likely through a “preferential” exclusion of solutes from proteins, which help to stabilize their structures (Gekko and Timasheff, 1981; Košťál et al., 2011a). In drosophilids, however, there is no clear evidence for substantial polyols accumulation correlated with cold hardiness (Kimura, 1982; Kely and Lee, 2001; Overgaard et al., 2007; Košťál et al., 2011a; Colinet et al., 2012).

Flies subjected to adult acclimation showed a low level of spermine (**Supplementary Figure S2**). Polyamines are involved in stress tolerance in plants (Gill and Tuteja, 2010) and putatively in insects (Michaud et al., 2008). Previous reports have detected increase in some polyamines (putrescine and cadaverine) in cold acclimated flies (Košťál et al., 2011a; Colinet et al., 2012). However, there is no report on variations of spermine levels in response to acclimation. Spermine is a polyamine formed from spermidine, and it has been shown to mediate stress resistance in *Drosophila* (Minois et al., 2012). It remains unclear why spermine was specifically at low levels in adult acclimated flies. Polyamine metabolism is very dynamic and low level of spermine may be due to synthesis of spermidine. Unfortunately, we could not detect this latter metabolite.

Flies from combined acclimation were also characterized by accumulation of several amino acids (Ala, Ile, Leu, Phe, Ser, and Val; **Supplementary Figure S1**) in comparison to control. Amino acids are known to possess cryoprotective properties. For example, Pro is responsible of increasing cold and freeze tolerance in *D. melanogaster* (Košťál et al., 2012), and it allows larvae from the fly *Chymomyza costata* to survive exposure to liquid nitrogen (Košťál et al., 2011c). Here, the concentration of Pro did not change dramatically in response to combined acclimation, while it increased in response to acclimation in *D. melanogaster* (Košťál et al., 2011a). The increased concentrations of the other amino acids were relatively small compared to changes observed with Pro in *C. costata* by Košťál et al. (2011c). In addition to Pro, many other free amino acids (mainly Arg, but also Ile, Leu, Val, and Ala) can promote flies' cold tolerance when supplemented in food (Košťál et al., 2016a). Although with different protocols, cold acclimation in *D. melanogaster* also triggered the accumulation of various amino acids in both adults (e.g., Val, Leu, Ser, Thr, Ile) (Colinet et al., 2012) and larvae (e.g., Pro, Asn, His, Glu) (Košťál et al., 2011a). In other insect species, cold acclimation or rapid cold hardening has consistently been correlated with the accumulation of various amino acids, among which Ala was often represented (Morgan and Chippendale, 1983; Storey, 1983; Hanzal and Jegorov, 1991; Fields et al., 1998; Michaud and Denlinger, 2007; Li et al., 2015). Here, Ala was the only amino acid showing the highest concentration after adult or combined acclimation (**Supplementary Figure S1**). The functional reasons for these amino acids accumulations

in cold-resistant phenotypes are not clearly understood, but it might relate to metabolic protection or stabilization of macromolecules (Yancey, 2005). Cold-protective mechanisms of free amino acids (against freezing mainly) have been extensively discussed by Košťál et al. (2016a) who suggested a range of different interactions, such as preferential exclusion, protection of native protein structure, binding of partially unfolded proteins, stabilization of membrane structure and vitrification. Apart from these cold-protective functions, many amino acids are components of biosynthetic pathways linked to glycolysis and TCA cycle; therefore, these changes may also reflect consequences of perturbations of the metabolic rate that often characterize acclimation (Lu et al., 2014; Li et al., 2015). Similarly, Košťál et al. (2011b) observed a moderate accumulation of amino acids, such as Pro, Gln, and Ala, in overwintering *Pyrhcoris apterus*, which probably resulted from a decrease of TCA cycle turnover rate, due to metabolic alteration induced by cold temperatures.

Here, we found that flies submitted to adult and combined acclimation were characterized by higher amounts of TCA intermediates than control flies or flies acclimated during development only (**Figure 3**). This could be linked to changes in metabolic rate. Indeed, metabolic cold adaptation or temperature compensation theories presume that at a same temperature, cold hardy insects showed similar or even higher metabolic rate than cold sensitive individuals (Hazel and Prosser, 1974; Chown and Gaston, 1999). Even if these theories are not always supported, several studies showed that cold acclimated insects had higher metabolic rate than warm acclimated ones (Terblanche et al., 2005; Isobe et al., 2013) and this may explain, at least in part, the changes in levels of TCA intermediates.

Despite showing unique metabolite, flies from developmental acclimation did not show drastic changes in their metabolite composition as observed in flies from combined and adult acclimation. Cold acclimation is a reversible trait (Slotsbo et al., 2016), therefore the moderate difference between flies from developmental acclimation and controls may be due to deacclimation. Despite these light metabolite divergences, our data showed that flies from developmental acclimation were more cold tolerant than control, based on all tested proxies. This suggests that physiological changes occurring during development, not only metabolic adjustments, persists and even carried over in adult stage, even if temperature returns permissive at adult stage.

Temporal Trajectories of Metatypes After Acute Cold Stress

Maintenance of metabolic homeostasis has been repeatedly associated with thermal tolerance in *D. melanogaster* (Malmendal et al., 2006; Colinet et al., 2012; Williams et al., 2014), although the functional mechanisms behind this correlated pattern are unknown and likely highly complex. We thus expected signs of metabolic inertia in cold acclimated phenotypes and signs of metabolic deregulation in chill-susceptible flies. Temporal analysis of metatypes revealed different temporal

patterns and mirrored cold tolerance of the four phenotypes (Figure 6). Control flies (the least cold tolerant) described a large and persistent metabolic deviation from initial state, suggesting a loss of metabolic homeostasis. Conversely, the most cold tolerant flies (flies from combined acclimation) showed only light initial deviation of metabolic trajectories followed by a rapid return to initial state during recovery period, suggesting a metabolic robustness and an efficient homeostatic response. Flies submitted to developmental and adult acclimation had intermediate cold tolerance and showed intermediate temporal metabolic response: initial deviation followed by incomplete return to initial metabolic state. Loss of homeostasis is likely related to development of thermal injuries (Malmendal et al., 2006) and several studies reported similar chill-induced homeostatic disruption in insects (Overgaard et al., 2007; Colinet et al., 2012; Teets et al., 2012; Williams et al., 2014).

Effects of chilling injuries can be immediate, accumulative or latently expressed (e.g., delayed mortality) (Turnock and Bodnaryk, 1991; Leopold, 2000; Overgaard et al., 2005). We noted that mortality after acute cold stress increased gradually with time of observation in control and flies acclimated during development suggesting latent damages. In contrast, flies that have been subjected to adult acclimation, combined or not with developmental acclimation, showed high and constant survival rates. Temporal increase of chill injuries in control and flies acclimated during development may be correlated with the temporal metabolic disorders that presented the highest distortion amplitudes in these two phenotypes.

The first axis of PCA, which described the temporal deviation of metabolic profiles, was correlated with accumulation of several amino acids, including several essential amino acids (Ile, Leu, Phe, Thr, and Val, Figure 6). Furthermore, the most chill-susceptible flies (control and developmental acclimation) were characterized by a temporal augmentation of the global amino acids pool (Figure 5). Such accumulation of amino acids after a cold stress has been reported in several insect species and is assumed to relate to protein breakdown, especially for essential amino acids, which can't be synthesized *de novo* (Lalouette et al., 2007; Overgaard et al., 2007; Košťál et al., 2011b; Colinet et al., 2012). However, direct evidence of cold-induced protein degradation is scarce (Hochachka and Somero, 2002).

Chill-susceptible phenotypes (mainly control flies and to a lesser extent flies from developmental acclimation) were characterized by temporal accumulation of several polyols (e.g., erythritol, sorbitol, and inositol) after the acute cold stress (Supplementary Figure S10). As discussed previously, accumulation of polyols after acclimation or rapid cold hardening may have protective functions (Walters et al., 2009; Crosthwaite et al., 2011). Increase in polyol concentrations after a cold stress is also a general response in insects (Yoder et al., 2006; Lalouette et al., 2007; Michaud et al., 2008; Colinet et al., 2012). So far, it is not clear whether these accumulations have protective values or whether they represent biomarkers of complex metabolic deregulations due to cold stress (Colinet et al., 2012). Since only the two least cold-tolerant phenotypes (flies from control and

developmental acclimation) displayed post-stress temporal increase in the level of some polyols, we can speculate that this most likely reflects a degenerative rather than a protective syndrome.

Concentration of amino acid pool didn't change in time after the cold stress in flies from combined acclimation (Figure 5), suggesting low magnitude of cold injuries to proteins structures. Conversely, global carbohydrates and TCA intermediates pools decreased with time in these flies (Figure 5). This may relate to rapid mobilization of energetic metabolism during recovery. Williams et al. (2016) reported that *D. melanogaster* lines selected for cold tolerance presented metabolic rate depression during cold stress, but that metabolic rate increased in a higher proportion than in chill-susceptible flies during recovery from a cold exposure at 0°C. In the beetle *Alphitobius diaperinus*, recovery from cold stress during thermal fluctuation is also associated with an important metabolic rate augmentation (Lalouette et al., 2011). Repairing chilling injuries and re-establishing homeostasis likely induces energetic cost (MacMillan et al., 2012); therefore, reduction of TCA intermediates in flies from combined acclimation may be related to energy requirement for recovery.

CONCLUSION

This study revealed that combining both developmental and adult cold acclimation resulted in a particularly high expression of cold tolerance in *D. suzukii*. This fly is believed to overwinter on wooded areas under leaf litters (Kanzawa, 1936), and trappings in autumn and winter revealed that captured flies were bigger, darker and more cold tolerant than flies captured in summer (Shearer et al., 2016). During these periods, flies are maybe developing slowly in infected fruits (protected in the litter) and then cold-acclimate as adults. Emerging flies by the end of winter season may therefore express high level of cold survival (at temperatures as low as -5°C) in the field. Our results indicate that cold tolerance plasticity of *D. suzukii* relies on physiological strategies similar to other drosophilids. Indeed, as frequently found in others drosophilids (e.g., Košťál et al., 2011a; Colinet and Hoffmann, 2012), we found that cold-acclimated *D. suzukii* accumulated low levels of cryoprotectants, such as sugars and amino acids, and were able to maintain metabolic homeostasis following cold stress. We highlighted that different acclimation treatments resulted in clearly distinct metabolotypes, suggesting that physiological responses highly depend on thermal history. Collectively, these data contribute to the emerging understanding of the physiological strategies used by *D. suzukii* to acquire cold tolerance. The present metabolic analyses provided correlative but not causative effects of cold acclimation. Artificial variations of the candidate metabolites, for instance by diet supplementation or by disrupting their biosynthesis, could shed light on the function(s) of these metabolites in cold tolerance acquisition. In addition to metabolic adjustments and mobilization of cryoprotectants, cold tolerance plasticity relies on many other mechanisms and pathways, such as

phospholipidic remodeling, maintenance of contractile function or altered thermal sensitivity of ion channel kinetics (Overgaard and MacMillan, 2016). Future studies should also explore these processes to gain better understanding of cold adaptation of *D. suzukii*.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors on request, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

TE and HC designed the experimental plan. TE conducted all experiments under the supervision of HC, DR, and MC. TE and HC analyzed the data and performed statistical analysis. TE and HC drafted the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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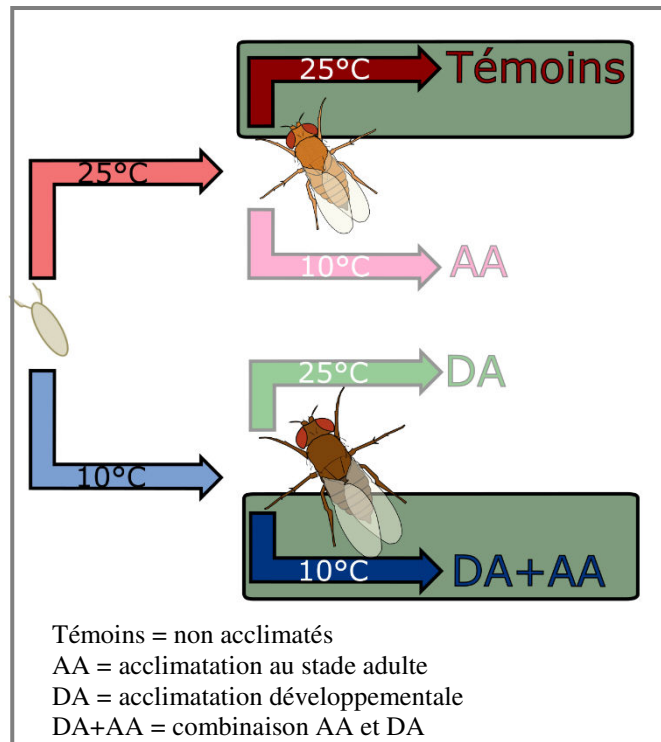
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Article 4: Cold acclimation triggers lipidomic and metabolic adjustments in the spotted wing drosophila *Drosophila suzukii* (Matsumara)

Résumé de l'article en français :

Les stress froids chroniques sont délétères pour les insectes intolérants au froid, et peuvent induire des dégâts physiologiques importants. Les insectes disposent de nombreuses capacités d'ajustements physiologiques permettant de contrecarrer les effets du froid, tels que le remodelage des membranes cellulaires ou la synthèse de cryoprotecteurs. La tolérance au froid est un élément clef déterminant la niche fondamentale d'un organisme. *Drosophila suzukii* est une espèce invasive originaire d'Asie de l'Est, par conséquent des connaissances concernant sa thermobiologie sont requises afin de mieux estimer son aire de répartition potentielle. Les mécanismes physiologiques sous-jacents à la plasticité de la tolérance au froid de cette espèce sont encore peu compris. Dans cette étude, nous avons exploré les modifications lipidiques et métaboliques associées avec l'acquisition de tolérance au froid chez cette drosophile par l'utilisation de techniques « omiques » (LC- et GC-MS/MS). Chez les mâles et les femelles acclimatées, nous avons observé des modifications en accord avec la théorie de l'adaptation homéoviscieuse/homéophasique des membranes : remaniement des têtes polaires des phospholipides et augmentation du degré d'insaturation des acides gras. L'augmentation de l'insaturation des acides gras a été également observée au niveau des triglycérides, probablement liée à une augmentation de l'accessibilité des réserves lipidiques. Par la suite, nous avons observé des modifications des profils métaboliques. En effet, les métabotypes des drosophiles acclimatées montraient une accumulation de plusieurs cryoprotecteurs potentiels (sucres et acides aminés). L'analyse des voies métaboliques suggérait un remodelage de plusieurs processus, incluant le métabolisme des purines, et la biosynthèse des aminoacyl-ARNt. Ces données fournissent une caractérisation à large échelle des remaniements lipidiques et des modifications des voies métaboliques en réponse à l'acclimatation chez *D. suzukii*, contribuant ainsi à la caractérisation des stratégies qui modulent la tolérance au froid de cette espèce.



Informations supplémentaires :

Compte tenu de leurs tailles imposantes, les figures et tableaux supplémentaires mentionnés dans cet article ne sont pas disponibles en annexes, mais sont accessibles en suivant les liens ci-dessous :

- Fig S1: <https://figshare.com/s/f386bdb731eec670699f>
- Supplementary Tables: <https://figshare.com/s/314073a828454cbb3838>
- Supplementary data set: <https://figshare.com/s/6ac157cfe6345010c886>

Cold acclimation triggers lipidomic and metabolic adjustments in the spotted wing drosophila *Drosophila suzukii* (Matsumara)

Under minor revisions at ‘American Journal of Physiology: Regulatory, Integrative, and Comparative Physiology’

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Running head

Lipidomic and metabolic changes in acclimated *D. suzukii*

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Author contributions statement

TE and HC designed the experimental plan. TE conducted all experiments under the supervision of HC. TE analysed the data and performed statistical analysis. TE and HC drafted and reviewed the manuscript.

Abstract

Chronic cold exposure is detrimental to chill susceptible insects that may accumulate chill injuries. To cope with deleterious effects of cold temperature, insects use various physiological strategies and metabolic adjustments, such as production of cryoprotectants, or remodeling of cellular membranes. Cold tolerance is a key element determining the fundamental niche of species. Since *Drosophila suzukii* is an invasive fruit pest, originating from East Asia, knowledge about its thermal biology is highly required. Physiological mechanisms underlying cold tolerance plasticity remains poorly understood in this species. Here, we explored metabolic and lipidomic modifications associated with cold tolerance acquisition in *D. suzukii* using Omics technologies (LC- and GC-MS/MS). In both acclimated males and females, we observed physiological changes fitting with homeoviscous/homeophasic adaptation of membranes: reshuffling of phospholipid head groups and increasing unsaturation rate of fatty acids.

Modification of fatty acids unsaturation were also observed in triacylglycerides, likely to increase accessibility of lipid reserves. At the metabolic level, we observed clear-cut differentiation of metabolic profiles, with acclimated metabotypes showing accumulation of several potential cryoprotectants (sugars and amino acids). Metabolic pathway analyses suggested remodeling of various processes including purine metabolism and aminoacyl tRNA biosynthesis. These data provide a large-scale characterization of lipids rearrangements and metabolic pathway modifications in response to cold acclimation in *D. sukikii*, and contribute to characterizing strategies used by this species to modulate cold tolerance.

Keywords

Cold tolerance, thermal plasticity, lipids, metabolic profiles, homeoviscous adaptation.

Introduction

Due to their viscous character, cellular membranes are thermosensitive structures (7). Extreme low temperatures can compromise their integrity by inducing transitions of membrane phospholipid (PL) bilayer from a fluid liquid crystalline phase to a more rigid lamellar gel phase (53). These changes in membrane fluidity mechanically alter activity and functions of membrane-bound enzymes (18, 41, 53), and may contribute to symptomatic loss of hydric and ionic homeostasis across membrane bilayers at cold (59, 60, 69, 73). Consequences might include neuromuscular dysfunctions, chill coma, and in most extreme cases death (44, 63, 72, 108). This is especially true for poikilothermic organisms, like insects, as their temperature directly depend on the environment, and for which stressful thermal events, like cold shocks, can severely impair physiological functions (23).

Ectotherms are capable of surviving stressful low temperatures using physiological plasticity such as cold acclimation (*i.e.* acquired cold tolerance subsequent to pre-exposure to cold temperatures). One of the most conserved physiological responses of ectotherms to low temperature is the preservation of membrane fluidity, referred to as homeoviscous adaptation of cell membranes (41, 92), or avoidance of membrane phase transition, referred to as homeophasic adaptation (42). These adjustments rely on remodeling membranes' PL, for instance by restructuring the polar head groups, shortening the fatty acid (FA) chains, or increasing FA unsaturation (53). These modifications help maintaining membrane fluidity and functioning under cold temperature, by inducing modification of van der Waals forces within membrane PL bilayer (16, 64). Similar modifications occur in stored lipids such as

triacylglycerides (TAG) in response to cold (57, 77, 102). As TAG represent the major energy reserve of insects, they play an important role in overwintering and cold survival (36, 77, 91).

Cold acclimation is often correlated with recruitment of low molecular mass compounds (29, 56, 71, 103). These compatible osmolytes (traditionally sugars, polyols and amino acids) are well known for their colligative effects at high concentrations (95, 109), but they may also play roles at low concentrations, for instance by stabilizing structures and integrity of proteins and membrane bilayers (5, 8, 20, 107). Cold acclimation may also induce large-scale changes in many enzyme activities, and therefore, metabolic pathways are putatively remodeled (71), such as pathways of central metabolism (12, 65, 66, 90).

The spotted wing drosophila, *Drosophila suzukii*, is an alien species in Europe (6) as well as in North and South America (39, 62). Contrary to the common species *Drosophila melanogaster* which lays eggs in ripening fruits, *D. suzukii* females oviposit exclusively in mature fruits, that larvae consume. This fly is highly polyphagous and can exploit a large number of wild hosts (50), but also a wide variety of cultivated fruit crops, including stone fruits, causing important harvest losses and economic costs (32).

Drosophila suzukii overwinters as adult in Northern America (99) and Europe (84). According to the definition of cold tolerance strategies (79), this fly is considered as chill susceptible. Indeed, it does not tolerate mild chilling (21, 26, 51, 88), and dies at temperatures well above its supercooling point (45). The overwintering success of *D. suzukii* likely relies on two main strategies: 1) migration to favorable and buffered microhabitats such as forests ; indeed, underneath the forest mulch temperatures remain positive in winter (89, 111) and 2) cold tolerance acquisition through plastic responses (90). Several studies showed that this fly is able to enhance cold tolerance thanks to various acclimation types, such as rapid cold hardening (28, 101), adult gradual acclimation (45, 104), or developmental acclimation (90, 101, 105). Recently, acclimated *D. suzukii* flies have been showed to survive temperatures as cold as -7.5°C (94). Using RNA sequencing Shearer *et al.*, (90) showed that winter-acclimated phenotype of *D. suzukii* was characterized by up-regulation of genes implied in ion transport, carbohydrate metabolism and glycolysis. In a previous target time-series analysis (27), we investigated metabolic adjustments during cold acclimation in *D. suzukii* and showed that acclimation was associated with metabolic robustness during cold stress and recovery period. Despite recent progresses in understanding *D. suzukii* thermal biology, physiological strategies underlying its cold tolerance plasticity remain poorly understood. Consequently, the aim of the

present study was to investigate on the impact of cold acclimation on physiological traits of importance regarding insect cold tolerance: membrane's composition, lipid reserves and metabolic responses. Here, we combined developmental and adult acclimation to produce a cold tolerant phenotype and used lipidomic and metabolomic holistic approaches to explore physiological correlations associated with cold acclimation response in *D. suzukii*. We hypothesized that cold acclimation would increase *D. suzukii* cold tolerance and predicted that acclimation will induce major physiological adjustments such as phospholipid changes fitting with homeoviscous or homeophasic adaptations (41), remodeling of TAG's carbon chains to increase their accessibility (87, 102), and metabolic pathway reorganizations to allow energy homeostasis and support cryoprotectants synthesis (e.g. sugars and amino acids) (11, 27, 56, 58, 71, 106).

Material and methods

Flies rearing and acclimation protocol

The *D. suzukii* line used in our experiments originated from wild flies collected from infested blueberries and raspberries in Thorigné Fouillard, France (48.0616N, -1.2387E) in September 2016. Flies were maintained under laboratory conditions for approximately 20 generations before experiments. Flies were reared in glass bottles (100 mL) and supplied with a carrot-based food (in 1 liter of water: agar: 15 g, sucrose: 50 g, carrot powder: 50 g, brewer yeast: 30 g, cornmeal: 20 g, kalmus: 8 g, Nipagin: 8 mL). Control flies were produced by placing bottles containing eggs in an incubator (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan) at 25°C, and a light/dark cycle of 12L : 12D. After emergence, adults were maintained at 25°C for 7 days. To generate cold acclimated flies, control adult flies (approximately 50) were allowed to lay eggs in bottles for 24 h at 25°C, then bottles with eggs were placed at 10°C (10L : 14D, same reference of incubator) to allow their development to the adult stage. After emergence, adults were kept at 10°C for 7 days. Consequently, flies were cold acclimated during both development and at adult stage. Seven-day-old control or cold-acclimated flies randomly taken from the rearing stock were used in experiments. All experiments were conducted on both females and males. Males were visually separated from females with an aspirator without CO₂ to avoid stress due to anesthesia (13).

Acute stress

From each treatment group flies were randomly taken and distributed in 10 replicates of 10 males or 10 females and submitted to -5°C for 100 min, using glass vials immersed in a glycol solution cooled by a cryostat (Cryostat Lauda ECO RE 630). After exposure, flies were allowed to recover in 40 mL food vials at 25°C (12L : 12D). Survival was assessed by counting the number of dead and living individuals in each vial 4 h, 24 h and 48 h after cold stress.

Critical thermal minimum ($C_{t_{min}}$)

To estimate the *D. suzukii* $C_{t_{min}}$ we used a glass knockdown column. It consisted in a vertical jacketed glass column (52 x 4.7 cm) containing several cleats to help flies not fall out the column while still awake. The column was linked to a cryostat (Cryostat Lauda ECO RE 630) to regulate temperature at 18°C. Temperature inside the column was checked using a thermocouple K, placed at mid height in the center of the column, connected to a Comark Tempscan C8600 scanning thermometer (Comark Instruments, Norwich, Norfolk, UK). Approximately 60 flies of each conditions were introduced at the top of the column. Flies were allowed to equilibrate in the device for a few minutes, and then the temperature was decreased to -5°C at 0.5°C/min. At each fly passing out and falling out of the column the $C_{t_{min}}$ (°C) was recorded.

Chill coma recovery time (CCRT)

CCRT is defined as the resurgence time of motor activity after a cold knockdown (22). In order to induce chill coma, we subjected 40 control and 40 acclimated flies of both genders to 0°C for 12 h, using food vials placed in a cooled-incubator (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan). Right after exposure, flies were rapidly transferred in a 25°C regulated room, and scattered individually on a large plane surface. At each fly able to stand up recovery time was recorded. Experimentation ended after 120 min, and non-recovered flies were counted.

Omics analyses

For each treatment and gender, five independent replicates (each consisting of a pool of approximately 40 seven days old flies) were snap-frozen in liquid N₂. Frozen samples were then sent to MetaSysX GmbH (Am Mühlenberg 11, 14476, Potsdam-Golm, Germany), where a coupled lipidomic and metabolomic non-targeted analysis was performed. Samples' extraction was performed according to metaSysX standard procedure, a modified protocol from Giavalisco *et al.*, (31).

LC-MS Measurements, data processing and annotation (Hydrophilic and Lipophilic Analytes)

The samples were measured with a Waters ACQUITY Reversed Phase Ultra Performance Liquid Chromatography (RP-UPLC, Waters, Milford, USA) coupled to a Thermo-Fisher Q-Exactive mass spectrometer which consists of an ElectroSpray Ionization source (ESI) and an Orbitrap mass analyzer (Thermo Fisher Scientific, Bremen, Germany). C8 and C18 columns were used for the chromatographic separation of lipophilic and hydrophilic compounds, respectively. The mass spectra were acquired in full scan MS positive and negative modes (Mass Range [100–1500]).

Extraction of the LC-MS data was accomplished with the software REFINER MS® 10.5 (GeneData, <http://www.genedata.com>). Alignment and filtration of the LC-MS data were completed using in-house metaSysX software. After extraction of the peak list from the chromatograms, the data were processed, aligned and filtered. Only features which were present in at least four out of the five replicates of at least one of the sample groups were selected. At this stage an average retention time (RT) and an average mass-to-charge ratio (m/z) values are given to the features. The alignment was performed for each platform independently (polar phase positive mode, polar phase negative mode, lipophilic phase positive mode, and lipophilic phase negative mode).

Data alignment was followed by the application of various filters in order to refine the dataset, among them removal of isotopic peaks, removal of in-source fragments of the analytes (due to the ionization method) and removal of additional lower intense adducts of the same analyte to guarantee the quality of the data for further statistical analyses. The annotation of the sample content was accomplished by matching extracted data from chromatograms with metaSyX library of reference compounds and with metaSyX database of lipids in terms of accurate mass and retention time.

MS/MS Lipid Annotation

Chromatograms were recorded in dd-MS2 Top 3 mode (Data Dependent tandem mass spectrometry) with the settings: Full Scan MS mode (Mass Range [100–1500]), NCE 25 (Normalized Collision Energy). Acyl composition of di- and triacylglycerids was established from the $[M+H]^+$ precursor ion fragmentation with detection of $[Acyl+NH_4]^+$ neutral losses in positive ion mode with further combinatorial calculation of the acyl composition. Acyl composition of phosphoglycerolipids was determined from the detection of $[Acyl-H]-$

fragments of the corresponding precursors in negative ion mode. Acyl composition of sphingolipids was established from the fragmentation pattern of [M+H]⁺ precursor ion in positive ionization mode.

GC-MS Measurements data processing and annotation

The samples were measured on an *Agilent* Technologies GC coupled to a *Leco Pegasus HT* mass spectrometer which consists of an electron impact ionization source and a time of fly (TOF) mass analyzer (*Leco*, St. Joseph, MI, USA). Column: 30 meters DB35; Starting temp: 85°C for 2 min; Gradient: 15°C per min up to 360°C. NetCDF files that were exported from the *Leco Pegasus* software were imported to “R”. The Bioconductor package *TargetSearch* was used to transform retention time to retention index (RI), to align the chromatograms, to extract the peaks, and to annotate them by comparing the spectra and the RI to the *Fiehn Library* and to a user created library. Annotation of peaks was manually confirmed in *Leco Pegasus*. Analytes were quantified using a unique mass.

Lipidomic and metabolomic data processing

Data from the five platforms were normalized to the sample fresh weight and to the median intensity of one thousand features (including all the annotated features and highly abundant features with unknown name) per replicate group. Subsequently, the normalized data of annotated features from all five platforms were merged to the final data matrix.

Statistical analyses

Cold tolerance

Analyses of acute cold survival and Ct_{min} were performed using R 3.4.3 (R Core Team, 2016). We modeled acute cold survival by specifying a generalized linear mixed-effects model (GLMM) with logistic link function for proportions outcome (i.e. number of dead/alive per vial). The response variable was dependent on the treatment (acclimation vs. control), gender, time to survival measurement, time x treatment and time x gender interactions. As survival rate was repeatedly measured after 4, 24 and 48 h post stress per vial, vial number was included in GLMM as random effect to account for repeated measures. We analyzed the effects of variables through an Analysis of Deviance (“Anova” function in “car” package, (30)). Ct_{min} values were compared using two-way ANOVA with treatment and gender as factors. CCRT data were analyzed using survival analyses with the software *GraphPad Prism5*. We made pairwise comparisons between each of the recovery curves using Gehan-Breslow-Wilcoxon tests. Alpha

level of significance for survival analyses was adjusted thanks to Bonferroni correction ($\alpha = 0.008$).

Lipidomic profiling

Lipidomic data were divided into three data sets: PL, TAG and other lipids (which include ceramides, free lipids and free FA). For PL and TAG data sets, between-class principal component analysis (PCA) (96) were run, using the “ade4” package in R (25). Monte Carlo tests were performed after each PCA to examine the significance of the difference among the classes (based on 1000 simulations). Next, we calculated the following indices for PL and TAG independently: unsaturation ratio (UFA/SFA, i.e. cumulative percent of all unsaturated FA [UFA] divided by the cumulative percent of all saturated FA [SFA]); ratio of polyunsaturation (PUFA/MUFA, i.e. cumulative percent of poly-unsaturated FA [PUFA] divided by the cumulative percent of mono-unsaturated FA [MUFA]); Unsaturation index (UI):

$$\sum \frac{n * (\% \text{ fatty acids with } n \text{ double bonds})}{100}$$

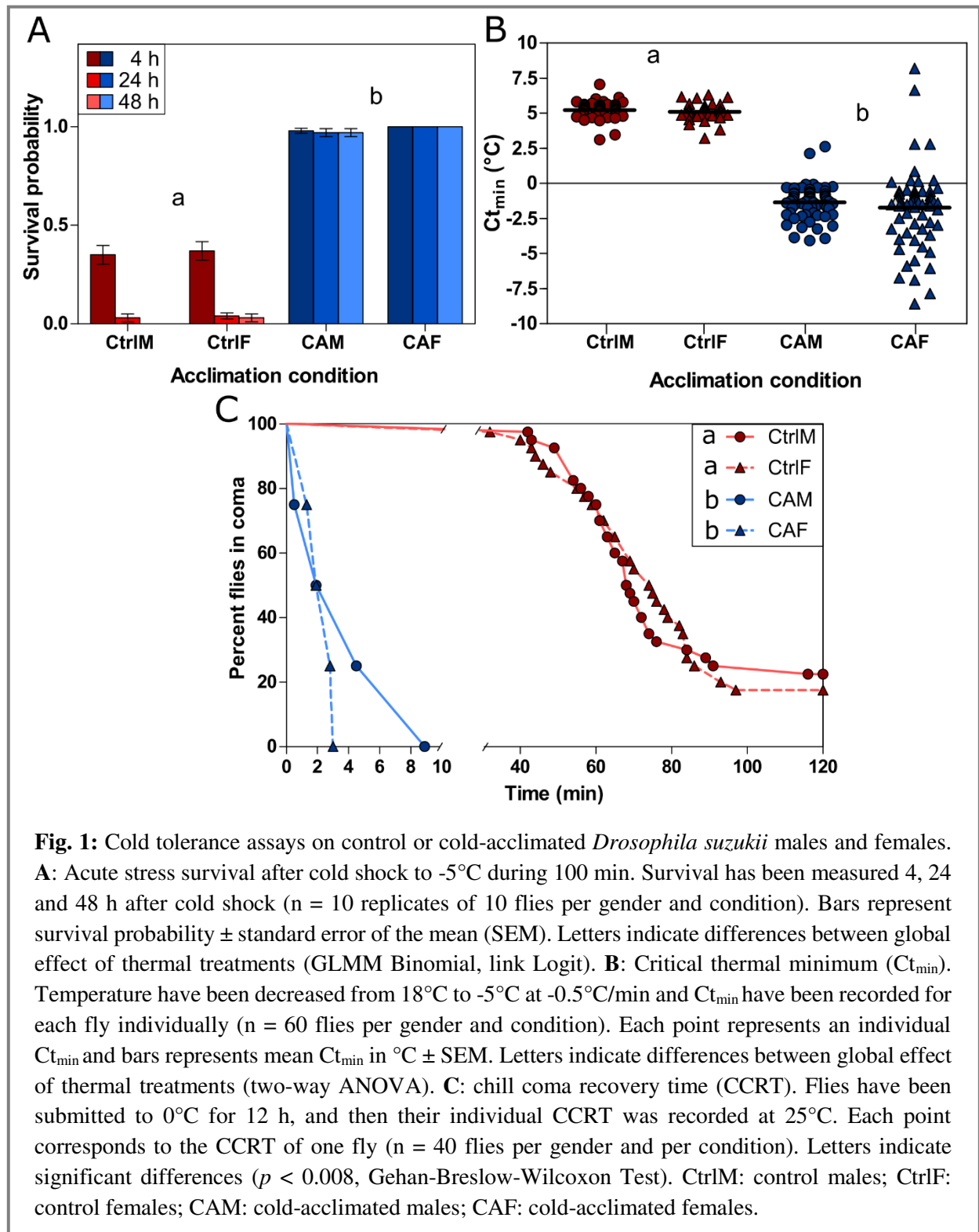
cumulative percent of all 16C FA divided by the cumulative percent of all 18C FA (ratio 16C/18C); cumulative percent of short FA ($\leq 16C$) divided by the cumulative percent of long FA ($> 16C$) (ratio short/long). Lipids composition and calculated indices were analysed in R, using two-way ANOVAs with gender, treatment (*i.e.* cold-acclimated vs. control) and their interaction as factors.

Metabolic profiling

Metabolic compositions of flies were compared using between-class PCA in the “ade4” package in R (25). Monte Carlo tests were performed to examine the significance of the difference among the classes (based on 1000 simulations). To identify the variables (*i.e.* metabolites) contributing the most to the PCA structure separation, the correlations to the principal components (PCs) were extracted and integrated into correlation circles.

In addition to multivariate analyses, we performed Student t-tests on each identified metabolite to compare mean relative abundances (*i.e.* normalized area under the curve values, AUC) between cold-acclimated and control flies, independently for males and females. The list of all significantly affected metabolites ($p < 0.05$) (available in Table S1) was used to run Metabolite Set Enrichment Analysis (MSEA) in Metaboanalyst 4.0 (10), based on *D. melanogaster* reference metabolome. Hypergeometric test was used as over representation analyses, and

relative-betweenness centrality was used to analyze pathway topology. To confirm and ensure consistency of MSEA results, we also used another pipeline (Reactome: <https://reactome.org/>) to conduct metabolic pathways analysis.



Results

Cold tolerance

Fig. 1A illustrates survival probability of control and cold-acclimated flies, 4, 24 and 48 h after cold shock. Acclimation promoted survival to -5°C in comparison to control conditions ($\chi^2 = 140.90$, $\text{df} = 1$, $p < 0.001$, Fig. 1A), leading to 100 % survival at 48 h after stress in cold-acclimated females. Globally survival decreased with time after stress ($\chi^2 = 41.67$, $\text{df} = 1$, $p < 0.001$, Fig. 1A), but this decrease was more pronounced in control flies in comparison to acclimated flies (treatment x time interaction: $\chi^2 = 14.88$, $\text{df} = 1$, $p < 0.001$, Fig. 1A). There was no difference between males and females' survival ($\chi^2 = 1.05$, $\text{df} = 1$, $p = 0.30$, Fig. 1A).

Fig. 1B represent mean Ct_{min} values. Mean Ct_{min} of acclimated flies was greatly lower than that of control flies ($-1.55 \pm 0.17^{\circ}\text{C}$ vs $5.16 \pm 0.05^{\circ}\text{C}$; $F_{(1,229)} = 966.14$, $p < 0.001$, Fig. 1B). Ct_{min} did not differ between males and females, and interaction between treatment and gender was not significant ($F_{(1,229)} = 1.61$, $p = 0.20$; $F_{(1,229)} = 0.384$, $p = 0.53$, respectively).

CCRT curves are displayed in Fig. 1C. In control flies, chill coma recovery progressively started after 40 min and 20% of males and females were still in coma after 120 min. Acclimated flies took only 3 and 9 min to recover for females and males, respectively (Fig. 1C). Consequently, there were significant differences between recovery curves of control and acclimated flies, for both genders (males: $\chi^2 = 64.00$, $\text{df} = 1$, $p < 0.008$; females: $\chi^2 = 64.00$, $\text{df} = 1$, $p < 0.008$). There were no differences between males and females in both treatment groups (control: $\chi^2 = 0.11$, $\text{df} = 1$, $p = 0.73$; cold-acclimated: $\chi^2 = 0.18$, $\text{df} = 1$, $p = 0.66$).

Omics analyses

Raw data from GC- and LC-MS/MS are available on the supplementary data set 1.

Lipidomic profiles

Coupled GC- and LC-MS/MS analyses resulted in 313 annotated lipid features. After discarding features containing NA values, we conserved 109 PL, 109 TAG and 51 other features (diacylglycerols, ceramides, free FA, free lipids). In subsequent analyses, we focused only on PL and TAG datasets. Fig. 2A and B present the results of the PCAs showing the ordination of classes within the first plane for PL and TAG separately. In both cases, the PCA revealed four clearly distinct and non-overlapping lipidotypes. Differences between control and acclimated flies were mainly explained by the first axis of PCAs ($\text{PC1} = 67.5\%$ of total inertia for PL, and

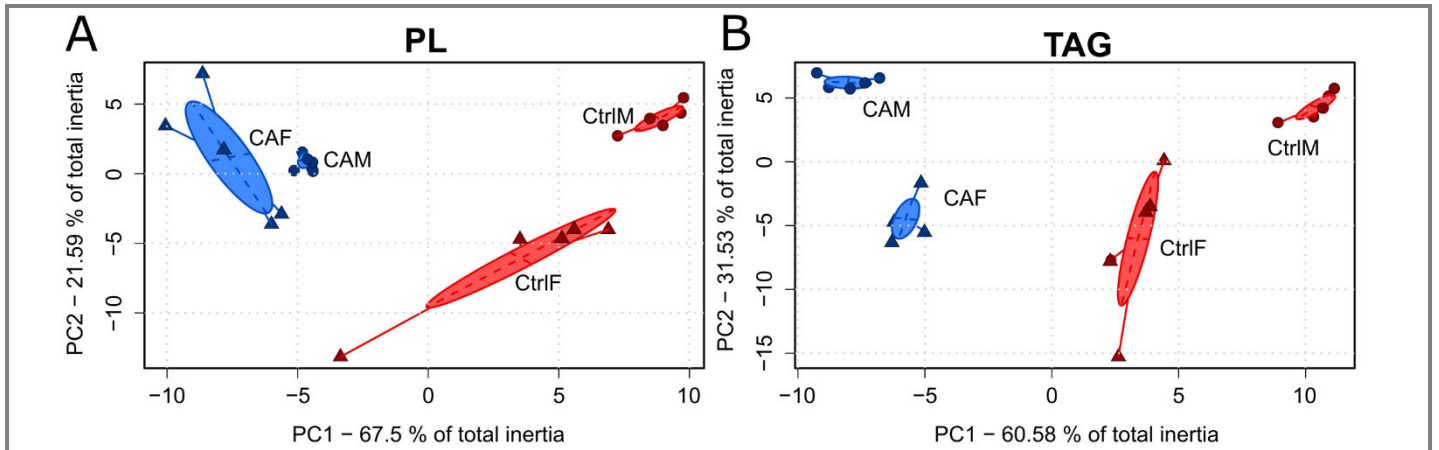


Fig. 2: Principal component analyses (PC1 vs. PC2) on **A:** the 109 phospholipids (PL) and **B:** the 109 triacylglycerides (TAG) identified in control and acclimated flies. Differences between lipidotypes were confirmed by Montecarlo randomizations (observed $p < 0.001$ for both PL and TAG). CtrlM: control males; CtrlF: control females; CAM: cold-acclimated males; CAF: cold-acclimated females.

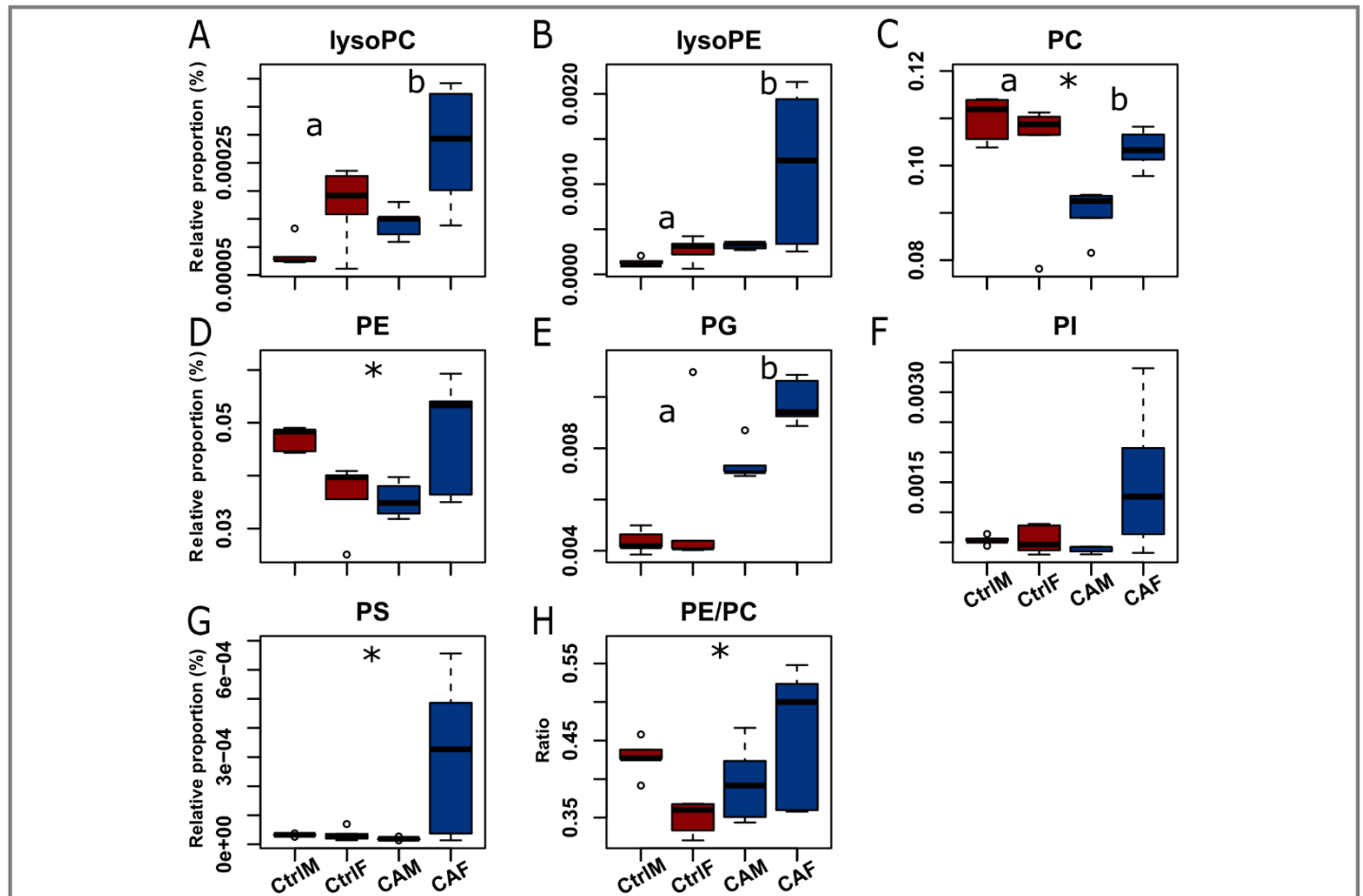


Fig. 3: A to G: Relative proportions of the different phospholipids' families after the different thermal treatments. **H:** ratio of the relative proportion of PE on PC. Letters indicate differences between global effect of thermal treatments and an asterisk indicates an interaction effect between genders and thermal treatments (two-way ANOVA). Open circles represent outliers. CtrlM: control males; CtrlF: control females; CAM: cold-acclimated males; CAF: cold-acclimated females; lysoPC: lyso phosphatidylcholine; lysoPE: lyso phosphatidylethanolamine; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PI: phosphatidylinositol; PS: phosphatidylserine.

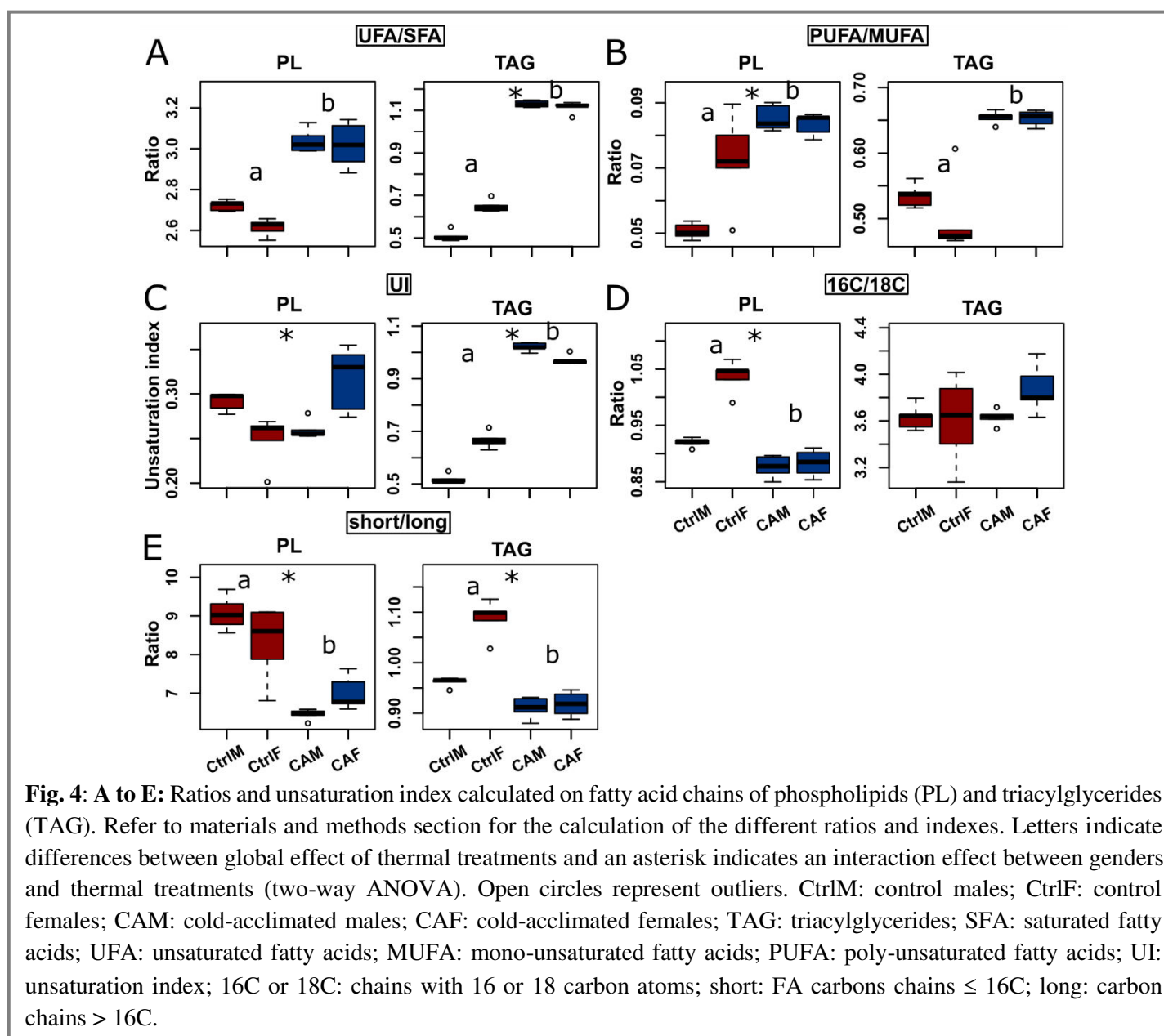
60.58% for TAG). Genders were differentiated along the second axis with 21.59% of total inertia for PL, and 31.53% for TAG (PC1 + PC2 = 89.39% for PL, and 92.11% for TAG). Correlation values of each lipidomic feature to PC1, PC2 and PC3 are available in Table S2 and S3 for PL and TAG respectively. Differences between lipidotypes were confirmed by Monte Carlo randomizations (observed $p < 0.001$ for both PL and TAG).

PL abbreviations of the different families are available in Table S4. Relative proportions of the different PL families according to thermal treatments and genders are shown in Fig. 3A to G. Relative proportions were calculated as the sum of AUC of each PL, divided by the sum of AUC of all lipid features. We also calculated the ratio of PE/PC, which is displayed in Fig. 3H. Cold-acclimated flies were characterized by a higher relative proportion of lysoPC, lysoPE and PG (Fig. 3A, B and E, $F_{(1,16)} = 6.91, p < 0.05$; $F_{(1,16)} = 7.78, p < 0.05$; $F_{(1,16)} = 24.99, p < 0.001$), while PC were more abundant in control flies (Fig. 3C, $F_{(1,16)} = 7.13, p < 0.05$). PS were relatively more abundant in cold-acclimated flies, but in females only (Fig. 3G, treatment x gender interaction: $F_{(1,16)} = 5.12, p < 0.05$). A similar difference was observed for PE in cold-acclimated females, while males followed the opposite trend (Fig. 3D, treatment x gender interaction: $F_{(1,16)} = 14.29, p < 0.01$). Consequently, this pattern reverberated on PE/PC ratio, which was higher in cold-acclimated females but lower in cold-acclimated males in comparison with controls (Fig. 3H, treatment x gender interaction: $F_{(1,16)} = 8.18, p < 0.05$). We also compared the relative proportions of TAG between cold-acclimated and treated flies, but we did not observe any difference (control females: $0.84 \pm 0.005\%$; control males: $0.82 \pm 0.002\%$; acclimated females: $0.82 \pm 0.006\%$; acclimated males: $0.85 \pm 0.002\%$. Effect of thermal treatment: $F_{(1,16)} = 1.10, p = 0.31$; Effect of gender: $F_{(1,16)} = 3.02, p = 0.10$).

Fatty acid compositions of carbon chains

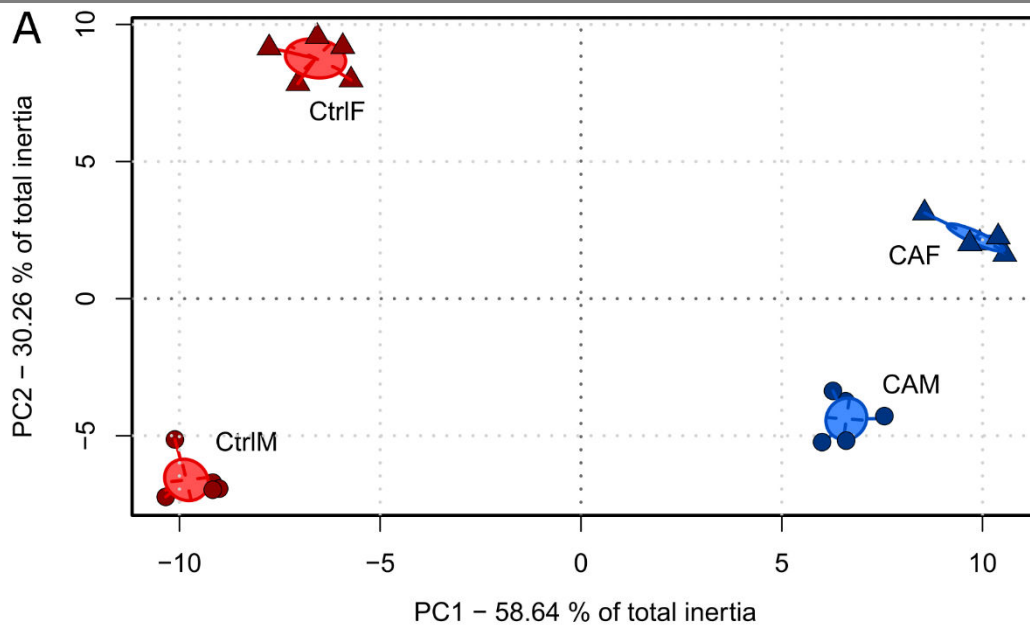
The metrics used to characterize FA compositions of PL and TAG are shown in panel Fig. 4. The list of abbreviations of these ratios is available in Table S4. Cold-acclimated flies had higher UFA/SFA ratio than control flies in both PL and TAG (Fig. 4A, $F_{(1,16)} = 143.20, p < 0.001$; $F_{(1,16)} = 2448.91, p < 0.001$, respectively). Likewise, acclimated flies had higher PUFA/MUFA ratio than controls in PL and TAG (Fig. 4B, $F_{(1,16)} = 89.51, p < 0.001$; $F_{(1,16)} = 43.48, p < 0.001$, respectively). UI was relatively higher in cold-acclimated than in control flies for TAG (Fig. 4C, $F_{(1,16)} = 1742.17, p < 0.001$), while for PL, it was higher only in cold-acclimated females, which resulted in significant treatment x gender interaction (Fig. 4C, $F_{(1,16)} = 21.63, p < 0.001$). Concerning the metrics on carbon chains lengths, ratios were globally lower

in cold-acclimated than in control flies, excepted in TAG which showed no variation in 16C/18C ratio (16C/18C: Fig. 4D, PL: $F_{(1,16)} = 104.35, p < 0.001$; TAG: $F_{(1,16)} = 1.80, p = 0.20$; Short/long: PL: Fig. 4E, $F_{(1,16)} = 97.95, p < 0.001$; TAG: $F_{(1,16)} = 56.86, p < 0.001$).

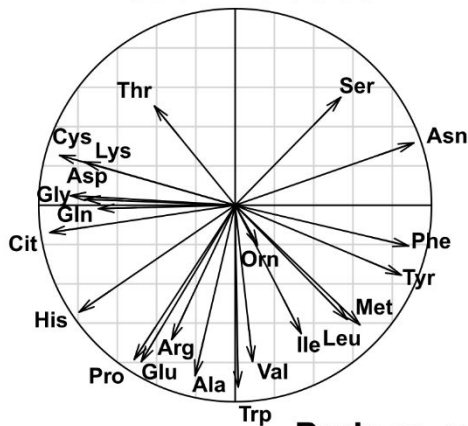


Metabolic profiling

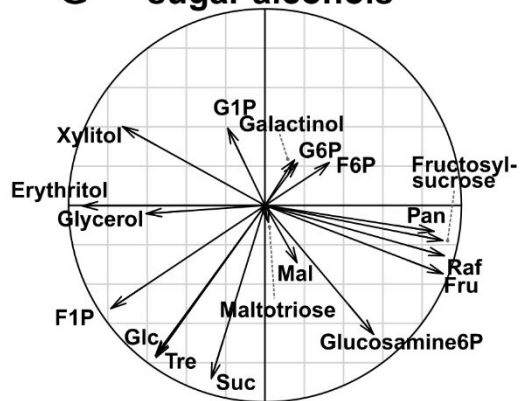
Coupled GC-MS/LC-MS analyses resulted in 312 metabolic features, among which 239 were identified. From these 239 metabolites, 53 were discarded because they contained missing values. The relative concentrations of the 186 remaining metabolites according to treatments are available in panel supplementary Fig. S1. Abbreviations as well as the chemical classes



B Free amino acids



C Carbohydrates and sugar alcohols



D Purines, pyrimidines and analogues

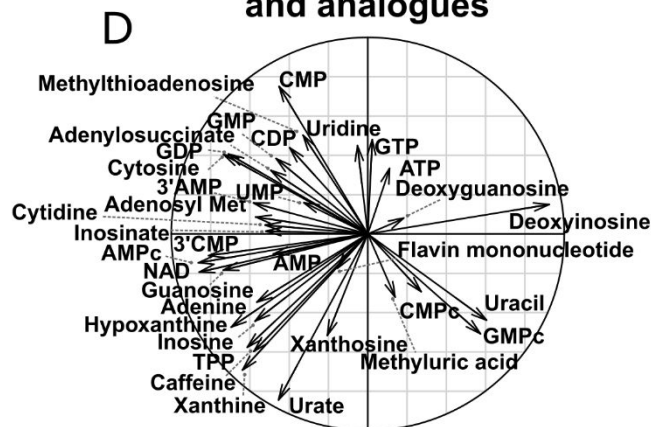


Fig. 5: **A:** Principal component analysis (PC1 vs. PC2) on the 186 metabolites identified in our control and acclimated flies. Differences between metabotypes were confirmed by Montecarlo randomizations (observed $p < 0.001$). Correlation circles from the principal component analysis on **B:** free amino acids; **C:** carbohydrates and sugar alcohols and **D:** purines, pyrimidines and analogues. CtrlM: control males; CtrlF: control females; CAM: cold-acclimated males; CAF: cold-acclimated females.

these metabolites belong to are displayed in Table S5. Chemical classes were attributed based on Human Metabolome Data Base (<http://www.hmdb.ca/>).

The PCA of metabolomic samples according to thermal treatments and genders is shown in Fig. 5A. The ordination of classes within the first plane of the PCA showed four distinct non-overlapping metabotypes (i.e. the metabolic compositions resulting from each phenotype).

Differences between acclimated and control flies were mainly explained by PC1 which accounted for 58.64 % of total inertia. Genders were opposed along PC2 which accounted for 30.26% of total inertia (PC1 + PC2 = 88.9 % of total inertia). Differences between metabotypes were confirmed by Monte Carlo randomizations (observed $p < 0.001$). Free amino acids, carbohydrates, and purine, pyrimidines and analogues were among the main metabolite classes of the dataset. To increase visibility, the projections of their variables into correlation circles are shown separately in Fig. 4B to 4D. These three correlation circles resulted from the same PCA. In addition, the list of all correlation values to PC1 and PC2 for the 186 metabolites are available in Table S5. The levels of several amino acids were positively correlated to PC1 (i.e., relatively more abundant in cold-acclimated flies) (Ser, Asn, Phe, Tyr, Met, Leu, etc.) (Fig. 4B). The relative levels of several sugars were also positively correlated to PC1, such as fructosylsucrose, Pan, Raf, Fru, while control flies were linked with several polyols (xylitol, erythritol, glycerol; Fig. 4C). Finally, the relative levels of the majority of purines and pyrimidines were negatively correlated to PC1 (i.e., relatively more abundant in controls) (Fig. 4D), and only a few metabolites of this class, such as deoxyinosine, uracil, or GMPC, were positively correlated to PC1.

Metabolite Set Enrichment Analysis (MSEA)

Univariate analyses showed that the relative level of 177 metabolites significantly differed between control and acclimated flies in males, and 112 in females (Table S1). To test for enrichment of biologically meaningful pathways, MSEA was performed in males and females separately, using the lists of differentially expressed compounds. MSEA identified eight significantly impacted pathways in males, and three in females. These pathways, as well as the metabolites involved, are displayed in Table 1. Two pathways were shared between males and females: aminoacyl-tRNA biosynthesis, and purine metabolism. These patterns were confirmed with Reactome pipeline and outcomes of these analyses are also available in Tables S6 and S7 for males and females, respectively.

Table 1: Outcomes from pathway analysis using Metaboanalyst 4.0

	Pathway	Total Hits	<i>p</i>	Impact	Decreased metabolites	Accumulated metabolites	
Males	Histidine metabolism	7	4	< 0.01	1	His; histamine; imidazoleacetic acid; methylhistidine	/
	Alanine, aspartate and glutamate metabolism	23	7	< 0.01	0.635	Asp; Gln; Glu; fumarate	arginiosuccinate; Asn; glucosamine 6-phosphate
	Ascorbate and aldarate metabolism	6	3	< 0.05	0.5	myo-inositol; D-glucuronic acid; D-saccharic acid	/
	Cysteine and methionine metabolism	25	6	< 0.05	0.496	L-cysteine; S-adenosyl methionine; 5'-methylthioadenosine	L-methionine; L-serine; L-Cystathionine
	Arginine and proline metabolism	37	10	< 0.01	0.294	Gln; Cit; Asp; Pro; Glu; S-adenosylmethionine; hydroxyproline; fumarate	argininosuccinate; urea
	Purine metabolism	64	14	< 0.01	0.147	GDP; xanthine; Gln; AMPc; AMP; deoxyadenosine monophosphate; hypoxanthine; inosine; urate; guanosine; GMPc; adenine; allantoate	deoxynosine; deoxyguanosine
	Aminoacyl-tRNA biosynthesis	67	15	< 0.01	0.137	His; Gln; Cys; Gly; Lys; Pro; Glu; Asp	Ser; Met; Asn; Phe; Ile; Leu; Tyr
Females	Starch and sucrose metabolism	17	6	< 0.01	0.129	Suc; Tre; Glc; D-glucuronic acid	Fru, Mal
	Taurine and hypotaurine metabolism	6	3	< 0.05	0.5	Cys; taurine; hypotaurine	/
	Purine metabolism	64	11	< 0.05	0.167	GDP; xanthine; AMPc; AMP; GMP; urate; guanosine; allantoate; adenylysuccinic acid	deoxynosine; deoxyguanosine
	Aminoacyl-tRNA biosynthesis	67	14	< 0.01	0.137	His; Cys; Gly; Asp; Lys; Pro; Glu	Asn; Phe; Ser; Met; Leu; Trp; Tyr

*Analysis has been performed on pooled down and up regulated metabolites. “Total” refers to the number of metabolites included in the respective pathways, and “hits” to the number of metabolites from the input list (Table S1) identified in the pathway. The *p* value resulted from Metabolite Set Enrichment Analysis (MSEA). “Impact” corresponds to the pathway impact value calculated from pathway topology analysis. List of metabolites identified in each pathway are separated depending on their fold change in cold-acclimated flies in comparison with controls (Decreased or increased). Males and females have been analyzed separately.*

Discussion

Cold acclimation increases cold tolerance of D. suzukii

As hypothesized, combined developmental and adult cold acclimation greatly promoted cold tolerance in both males and females, and this was consistently observed with all tested metrics (i.e., survival, CCRT and $C_{t_{min}}$. See Fig. 1). The basal cold survival of control flies measured after exposure to -5°C for 100 min was similar to data reported by Jakobs *et al.*, (45) and Everman *et al.*, (28) (i.e. 20% survival after 1 h at -7°C and 10% after 1 h at -6°C , respectively), and acclimation greatly promoted acute cold stress survival, as previously reported (105). Shorter CCRT after adult acclimation has also been reported by Jakobs *et al.*, (45). Finally, as observed here, Toxopeus *et al.*, (101) also observed a clear decrease in $C_{t_{min}}$ after combined developmental and adult acclimation.

Several studies reported that cold tolerance is highly plastic in this species (37), especially when flies are acclimated during both development and adult stage (90, 101, 105), which could in part explain its overwintering success. Furthermore, previous work showed that developmental acclimation of *D. suzukii* under laboratory conditions resulted in relatively bigger, darker and more cold tolerant flies, similar to individuals captured during winters in invaded areas (90, 101, 105). Recently, it was shown that laboratory maintenance does not alter ecological (stress tolerance) and physiological patterns of *Drosophila* species (68). Yet, caution should be exercised in the extrapolation of these data to field reality because results were obtained from a laboratory-adapted line exposed to controlled thermal environment. In natural environment, insects may be exposed to multiple and repeated stresses that may affect patterns of cold tolerance (52).

Acclimation-driven phospholipidic readjustments

The mechanisms underlying cold tolerance acquisition in *D. suzukii* remain poorly understood and our goal here was to explore lipidomic readjustments driven by cold acclimation. Exposure to cold acclimation often induces marked alterations of lipid composition and of the physical properties (fluidity) of membranes, a response conserved among taxa (18, 33, 40, 41, 57, 83). We identified more than one hundred different PL. The PL composition of *D. suzukii* membranes was mainly dominated by PE and PC (see Table S2), similarly to *D. melanogaster* membranes composition (9, 15, 38, 46, 56). Cold acclimation induced significant changes in PL composition that resulted in a distinct differentiation among the lipidotypes (Fig. 2) and

reshuffled the PL headgroups (Fig. 3). The major changes induced by acclimation were observed both in males and females, although responses were sometimes gender-specific which might explain why males and females lipidotypes were non-overlapping. LysoPE and lysoPC were relatively more abundant in cold-acclimated males and females. Even if LysoPL represent only a minor proportion of membrane's PL, they seem to play important roles in membranes' response to cold temperatures in *D. melanogaster* (15). LysoPL have an inverted conical shape, which disrupts the tight packaging of PL membranes, increasing their fluidity (53). Their precise functions for cold tolerance however, remain to be clearly defined. Moreover, PG accumulated in response to cold acclimation in both genders, whereas PS accumulated in females only. PG are common PL of cell membranes, and play important roles in response to environmental variations and in membrane fluidity (67). PC decreased after acclimation in both genders while PE and the ratio PE/PC decreased in acclimated males and increased in females. PE/PC changes is a common response to cold temperatures in insects (33, 100). In males and females *Drosophila melanogaster* cold acclimation or fluctuating cold temperature led to an augmentation of the PE/PC ratio (15, 16, 83), but not rapid cold hardening (70). PC arbore a cylindrical form, which provides membranes a highly organized, compact and rigid structure (53). Consequently, decrease of PC at the expense of other PL is supposed to increase membrane fluidity (40, 53).

Lipids restructuring was also visible at the scale of PL's FA chains: we observed relative increases in the proportions of FA unsaturation and polyunsaturation after cold acclimation (Fig. 4A and B). As mentioned earlier, desaturation of FA is a common response to cold in poikilotherms (53) and insects (3, 49, 57). In *Drosophila* species, several studies reported no evidence of marked desaturation of FA in response to low temperatures (15, 16, 70, 76, 83); however, other studies reported increase in unsaturation ratios in response to cold selection (34) or temperature shift (17). Desaturation of FA is linked to fatty-acyl-CoA desaturases activity (19, 49). These enzymes insert *cis* double bonds into carbon chains of FA, resulting in a bend of approximately 30°. Therefore, UFA occupy more space into the PL bilayer than SFA, resulting in a decrease of the tight packing of PL and thus increasing membrane fluidity (53).

Fatty acids of PL were also characterized by an increase of carbon chain lengths after acclimation (decrease of 16C/18C and short/long ratios, Fig. 4D and E). Usually in insects, cold hardiness is linked with FA shortening (1, 2, 74), but there are several cases where the opposite pattern was observed (3, 4, 54, 85). In *Drosophila* species, the pattern is again variable, with

studies reporting increase (15, 76, 78, 82), decrease (83) or no change (81) in 16C/18C ratio in response to various low temperature treatments. These incongruities may result from the diversity of cold treatments applied and analyzed, ranging from short-term plastic responses to cold, to long-term cold adaptation. Košťál (53) suggested that desaturation of FA and shortening of carbon chain length, could be alternatively used by organisms to obtain a similar result: increase of membrane fluidity.

The overall PL adjustments resulting from gradual cold acclimation in *D. sukukii*, (PL headgroup modifications, and desaturation of FA) are typical responses fitting with homeoviscous adaptation, confirming our hypothesis. These modifications may contribute to maintenance of membrane fluidity and functions under cold temperature and partly explain the enhanced cold tolerance of acclimated flies.

In this work, we used an acclimation protocol similar to that of *Toxopeus et al.*, (101). These authors showed that female flies exposed to this acclimation had arrested ovarian development. Therefore, differences observed here between acclimated and control flies could also result, at least in part, from differences in reproductive status of flies (35). However, the fact that acclimation patterns were found in both males and females suggests that differential oogenesis was not a major confounding effect. Both lipidomic and metabolomics profiles change with age and aging (14, 35, 43, 86). Since physiological age of acclimated flies could slightly differ from that of control, this may partly account for differences between our treatment groups.

Changes in stored lipids

Cold acclimation did not induce change in the relative amount of TAG in *D. sukukii* males or females confirming previous results (101). However, we noticed an increase of the proportions of UFA and PUFA in TAG from cold-acclimated flies (Fig. 4A and B). TAG desaturation is commonly observed prior to or upon experiencing a decrease of body temperature in poikilotherms (41), including insects (3, 36, 57, 102) such as drosophilids (76, 77). TAG represent the major energy source in insects (36, 91), and low temperature, by increasing their viscosity, tend to hamper their accessibility for basal metabolism. As for desaturation of FA in membrane's PL, desaturation of TAG induce a decrease of their melting point, increasing fluidity and thus accessibility at cold temperatures (57, 87, 102).

Changes in metabolic composition

Through alteration of many metabolic pathways (Table 1), cold acclimation resulted in metabolotypes that were clearly distinct from those of non-acclimated counterparts in both males and females (Fig. 5A). Only two altered pathways were shared between cold-acclimated males and females: purine metabolism and aminoacyl-tRNA biosynthesis. The large majority of purines and pyrimidines were correlated with non-acclimated controls (Fig. 5D). Changes in purine metabolites and purine metabolism related to low temperatures in insects have been reported in previous studies (71, 106). In the psyllid *Diaphorina citri* for example, it was evidenced that after 5 days exposures at 35, 20 or 5°C the nucleotides and sugar-nucleotides were all correlated with warm temperatures, reflecting the pattern found in the present study. Purine and pyrimidine nucleotides have essential functions: building blocks of DNA and RNA, energy carriers, cell signaling molecules, and play central part in metabolism (47). Due to these multiple roles, it would be premature to speculate about the precise involvement of these molecules in cold acclimation. We can however assume that low temperature during acclimation may have affected intracellular nucleotide pools consequently to kinetic effects on metabolism.

Purines are also directly involved in transcription and translation (47). Therefore, the alteration of purine metabolism, as suggested by our data, could be linked to the alteration of the aminoacyl-tRNA biosynthesis pathway (Table 1). RNA sequencing revealed in *D. melanogaster* that aminoacyl-tRNA biosynthesis was the most altered pathway after cold acclimation (71). Similarly, in *D. sukuzii* RNA sequencing demonstrated that cold acclimation resulted in down regulation of several functions and processes involved in translation (90). All these modifications could be linked with a global decline of the translational machinery due to low temperature (98).

We hypothesized that cold acclimation would be correlated with mobilization of cryoprotectant molecules, specifically amino acids (e.g. Pro, Arg) and sugars (e.g. Glc or Tre). According to PCA, it appeared that metabolic profiles of cold-acclimated flies were correlated with relative increase in many amino acids (Ser, Asn, Phe, Tyr, Met, Leu, etc., See Fig. 5B). This was reflected in MSEA that detected involvement of pathways related to several amino acids in acclimated males. Amino acids are known to act as cryoprotectant in insects, especially Pro (56, 58, 61), Arg (55) and Ala (65, 75). However, here, changes in Ala and Pro levels were correlated with difference between genders but not thermal treatments (Fig. 5B), although their

metabolic pathways were significantly altered in males (Arg and Pro metabolism; Ala, Asp and Glu metabolism, see Table 1). Other amino acids are known to accumulate in response to cold in *D. melanogaster*, like Val, Leu, Ser, Thr, Ile, Asn, His or Glu (11, 56). In a previous target study (27) we observed a similar recruitment in amino acids after cold acclimation in *D. suzukii*. Except from Pro and Arg that have strong and well-defined cryoprotective roles in promoting cold/freeze tolerance in *Drosophila*, many other amino acids can also show mild positive effects at some concentrations, such as Val, Ile, Leu, Asn (55). Our previous results (27) showed that amino acids concentrations in acclimated flies did not exceed the nmol/mg⁻¹. At these concentrations it is unlikely that amino acids contributed to cold tolerance through colligative effects, but they could still act at low concentrations by protecting and stabilizing macromolecule structures (107).

We also observed changes in the relative level of several carbohydrates linked to cold-acclimated flies (e.g. Pan, Raf, Fru, Fructosylsucrose, Fig. 5C). Also, the starch and sucrose metabolism pathway was significantly altered in males (Table 1). Cold tolerance is associated with increased carbohydrates concentrations in various insects (24, 110) and also in *D. melanogaster* (11, 56, 80). Furthermore, in *D. suzukii* and *D. melanogaster* transcriptomic data suggest that carbohydrates and starch and sucrose metabolism pathways were altered by cold acclimation (71, 90). Tre is one the most commonly carbohydrates correlated with cold tolerance (48, 56, 95), but here, it was not linked with cold acclimated flies. In a previous work however, we showed that several carbohydrates accumulate in *D. suzukii* after cold acclimation, including Tre (27).

The protocol used in the present study was somewhat specific as it combined juvenile and adult acclimation. Physiological patterns may vary by using different acclimation procedures (stage, temperature or photoperiod for example). In addition, it is conceivable that metabolic remodeling observed after acclimation might result from non-adaptive changes that are non-essential for cold tolerance acquisition. Indeed, it is possible that some of these changes resulted for moderate low temperature during cold acclimation, as cold exposure is known to trigger metabolic alteration (11, 80, 97, 106). However, we previously showed that after a similar acclimation procedure, *D. suzukii* flies were able to maintain metabolic homeostasis after a cold stress, contrary to non-acclimated individuals (27).

Perspectives and significance

This study represents the first large-scale analysis of cold acclimation response in the invasive pest *D. suzukii* based on combined lipidomic and metabolic approaches. We showed that acquiring cold tolerance thanks to cold acclimation generated a phenotype that was clearly distinct from control at both lipidomic and metabolic levels. This suggests system-wide reprogramming in response to acclimation across various metabolic pathways and lipidic changes. We showed that acquired cold tolerance in *D. suzukii* correlated with homeoviscous adaptations of membrane PL, increased fluidity of stored lipids, and deep metabolic adjustments involving compatible solutes such as sugars and various amino acids. The acclimated phenotype resulting from our experiments was relatively similar to those of flies captured in the wild in autumn and winter. These winter flies probably resulted from larvae and pupae that developed in fruits in autumn (50). Hence, they may acclimate during both development and as adult (28, 84, 90, 93, 101, 105). Therefore, it is likely that wild *D. suzukii* can use physiological adjustments similar to the ones we described here to enhance their cold tolerance and successfully overwinter in invaded areas. Our data add novel information for *D. suzukii* cold physiology and participate to the understanding of its thermal biology. Future studies should analyze the physiological strategies used by *D. suzukii* during autumn and winter in nature to assess if they use similar strategies as laboratory cold-acclimated flies.

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Disclosures

The authors declare there are no competing interests.

Supplementary information

Supplementary Figure 1: <https://figshare.com/s/f386bdb731eec670699f>

Supplementary tables: <https://figshare.com/s/314073a828454cbb3838>

Supplementary data set 1: <https://figshare.com/s/6ac157cfe6345010c886>

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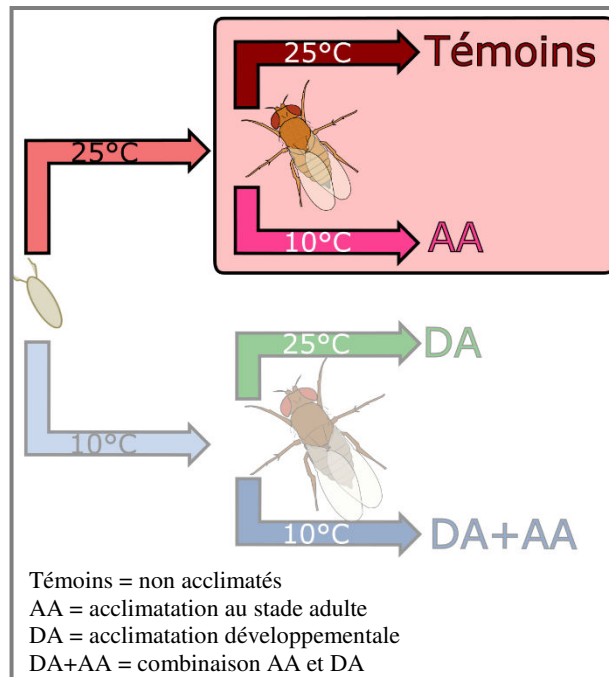
Article 5 : Cold acclimation triggers major transcriptional changes in *Drosophila suzukii*

Résumé de l'article en français :

Contexte – Les mécanismes physiologiques des insectes peuvent s'ajuster tout au long de leur vie leur permettant de faire face aux conditions thermiques délétères : c'est le principe du phénomène d'acclimatation thermique. Comme chez la plupart des espèces, l'acclimatation chez *Drosophila suzukii* augmente sa tolérance au froid. Toutefois, les mécanismes sous-jacents de cette plasticité ne sont pas connus chez cette espèce. Dans cette étude, nous avons tester la tolérance au froid des drosophiles en réponse à un gradient de durée d'acclimatation (*i.e.* des préexpositions allant de 2 h à 9 jours à 10°C), puis nous avons comparé les ajustements transcriptomiques (par séquençage d'ARN) des drosophiles acclimatées en comparaison aux drosophiles témoins.

Résultats – La tolérance au froid de *D. suzukii* augmentait avec la durée d'acclimatation. Les drosophiles les plus tolérantes au froid (après 9 jours d'acclimatation) ont été sélectionnées pour l'analyse transcriptomique. Au total, 2908 gènes étaient différentiellement exprimés : 1583 sur- et 1325 sous-régulés chez les drosophiles acclimatées. Les annotations fonctionnelles ont révélé de nombreux GO-term enrichis chez les drosophiles acclimatées, parmi lesquelles le transport d'ions à travers les membranes, et la signalisation cellulaire étaient fortement représentés. De même, l'activité neuronale et le métabolisme des carbohydrates étaient des GO-term enrichis après acclimatation. Ces résultats ont également permis de révéler que de nombreux GO-term liés à l'oogenèse étaient sous-représentés chez les drosophiles acclimatées.

Conclusions – L'implication d'un important cluster de gènes lié au transport des ions chez les drosophiles acclimatées suggère des ajustements de la capacité à maintenir l'homéostasie ionique et hydrique. Ces processus sont des mécanismes sous-jacents clés de la tolérance au froid chez les insectes. La sous-régulation des gènes liés à l'oogenèse chez les femelles acclimatées reflète probablement le fait qu'elles étaient maintenues à 10°C, une température bloquant l'oogenèse chez cette espèce. Globalement, ces résultats permettent de comprendre les mécanismes sous-jacents à l'acquisition de la tolérance au froid chez *D. suzukii*, des données d'importances pour comprendre le succès de son invasion sous nos latitudes.



Informations supplémentaires :

Les figures supplémentaires mentionnés dans cet article sont accessibles en **annexe 5**. Compte tenu de leurs tailles conséquentes, les tableaux supplémentaires sont disponibles en suivant ce lien : <https://figshare.com/s/3df7061e3ff5bed39304>

(Une version numérique des figures supplémentaires est également disponible en suivant le lien).

Cold acclimation triggers major transcriptional changes in *Drosophila suzukii*

Submitted to BMC Genomics

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Abstract:

Background

Insects have the capacity to adjust their physiological mechanisms during their lifetime to promote cold tolerance and cope with sublethal thermal conditions, a phenomenon referred to as thermal acclimation. The spotted wing drosophila, *Drosophila suzukii*, is an invasive fruit pest that, like many other species, enhances its thermotolerance in response to thermal acclimation. However, little is known about the underlying mechanisms of this plastic response. Here, we promoted flies' cold tolerance by gradually increasing acclimation duration (*i.e.* pre-exposure from 2 h to 9 days at 10°C), and then compared transcriptomic responses of cold hardy *versus* cold susceptible phenotypes using RNA sequencing.

Results

Cold tolerance of *D. suzukii* increased with acclimation duration; the longer the acclimation, the higher the cold tolerance. Cold-tolerant flies that were acclimated for 9 days were selected for transcriptomic analyses. RNA sequencing revealed a total of 2908 differentially expressed genes: 1583 were up- and 1325 were downregulated in cold acclimated flies. Functional annotation revealed many enriched GO-terms among which ionic transport across membranes and signaling were highly represented in acclimated flies. Neuronal activity and carbohydrate metabolism were also enriched GO-terms in acclimated flies. Results also revealed many GO-terms related to oogenesis which were underrepresented in acclimated flies.

Conclusions

Involvement of a large cluster of genes related to ion transport in cold acclimated flies suggests adjustments in the capacity to maintain ion and water homeostasis. These processes are key mechanisms underlying cold tolerance in insects. Down regulation of genes related to oogenesis in cold acclimated females likely reflects that females were conditioned at 10°C, a temperature that prevents oogenesis. Overall, these results help to understand the molecular underpinnings

of cold tolerance acquisition in *D. suzukii*. These data are of importance considering that the invasive success of *D. suzukii* in diverse climatic regions relates to its high thermal plasticity.

Keywords: thermal plasticity, cold tolerance, transcriptomics, genes expression, spotted wing drosophila

Background

The spotted wing drosophila, *Drosophila suzukii*, is a fruit fly originating from South-East Asia, invasive in Europe as well as North and South America [1] where it is continuously expanding its repartition area [2, 3]. Contrary to its relative species *Drosophila melanogaster*, which lays eggs exclusively on rotten fruits, *D. suzukii* females possess a serrated ovipositor that allows them to break through fruit skin and lay eggs in fresh mature fruits [4]. After hatching, the larvae consume and damage the fruits, causing damages that turn them uncommerciable [5, 6]. Furthermore, wounds caused by female's ovipositor offer a way of entrance for pathogens, causing secondary infections [6]. This fly is highly polyphagous, targeting a broad range of cultivated fruit crops [1, 5, 6], as well as wild hosts [7, 8]. Consequently, this pest has an important economic impact, especially for soft fruit production [1, 5, 6]. Several biological control strategies are currently under deep investigation to counter this pest [9–11]. In order to facilitate its management, knowledge about its biology is highly required, especially about its thermal physiology [1, 9, 12]. Indeed, thermal tolerance and especially the capacity of alien species to modulate their thermal tolerance thanks to phenotypic plasticity, is believed to be a key factor of their invasive success [13, 14]. Therefore, increasing knowledge about thermal biology of *D. suzukii* is essential to predict evolution of its invasion front or its population dynamics in invaded areas and facilitate its control.

Like most insect species [15], *D. suzukii* is chill susceptible which means that it rapidly suffers chilling injuries at temperatures well above its freezing point [16–19]. In insects, chilling injuries result from complex physiological alterations due to cold exposure such as loss of ion and water homeostasis which participate to the disruption of neuromuscular functions, leading to chill coma. Physiological injuries also compromise cell integrity, resulting in tissue damage, and in most extreme cases death [15, 20, 21]. To cope with these deleterious effects, insects can adjust their physiological state in anticipation of cold stress. Cold acclimation (triggered by pre-exposure to mild low temperature) is a typical example of phenotypical plasticity. Cold

acclimation induces deep and complex physiological remodeling such as changes in composition of membranes [22], mobilization of cryoprotective metabolites [23–26], maintenance of metabolic homeostasis [27–30], altered stress genes expression [31, 32], and enhanced ability to maintain ion and water balance [31, 33, 34]. These changes prevent the development of chilling injuries, resulting in increased cold tolerance [15].

Drosophila suzukii displays a high plasticity of cold tolerance and responds to all forms of acclimation such as rapid cold hardening [35, 36], adult acclimation [27, 37, 38] and developmental acclimation [27, 36, 39, 40]. This fly is capable of surviving a three days exposure at -7.5°C after dynamic acclimation (i.e. gradual cooling) [41]. Despite the economic importance of this species, the number of studies that focused on the underlying mechanisms of this plasticity remains scarce. Shearer *et al.*, [39] explored transcriptional adjustments associated with the winter-phenotype generated by a combination of developmental and adult acclimation. This thermal treatment results in a cold tolerant winter phenotype showing a reproductive dormancy. In a previous work [27], we showed that flies subjected to both developmental and adult acclimation were characterized by accumulation of cryoprotectants and were able to maintain metabolic homeostasis after cold stress, confirming a deep biochemical remodeling linked to acclimation. So far, there is a limited understanding of the molecular mechanisms that underlie cold tolerance plasticity of *D. suzukii*.

In the present study, we subjected mature adults of *D. suzukii* to increasing acclimation periods (pretreatment from 2 hours to 9 days at 10°C) in order to investigate the cold pre-exposure period needed to reach high cold tolerance. Next, we identified the molecular correlates underlying cold tolerance acquisition in *D. suzukii* using the hypothesis-generating and explorative power of RNA sequencing (RNAseq). We expected to find regulations of candidate gene sets involved in the canonical cold-acclimation mechanisms, such as membrane remodeling, cryoprotectant (sugar) metabolism, ionic/water balance or stress proteins.

Results

Cold tolerance

Survival to acute (-5°C for 1 h) and to chronic cold stress (0°C for 24 h) as a function of acclimation is displayed in Fig. 1a and b, respectively. Regardless of sex, cold survival reached a maximum after 144 h (6 days) of acclimation (Fig. 1a and b). Both acute or chronic cold stress

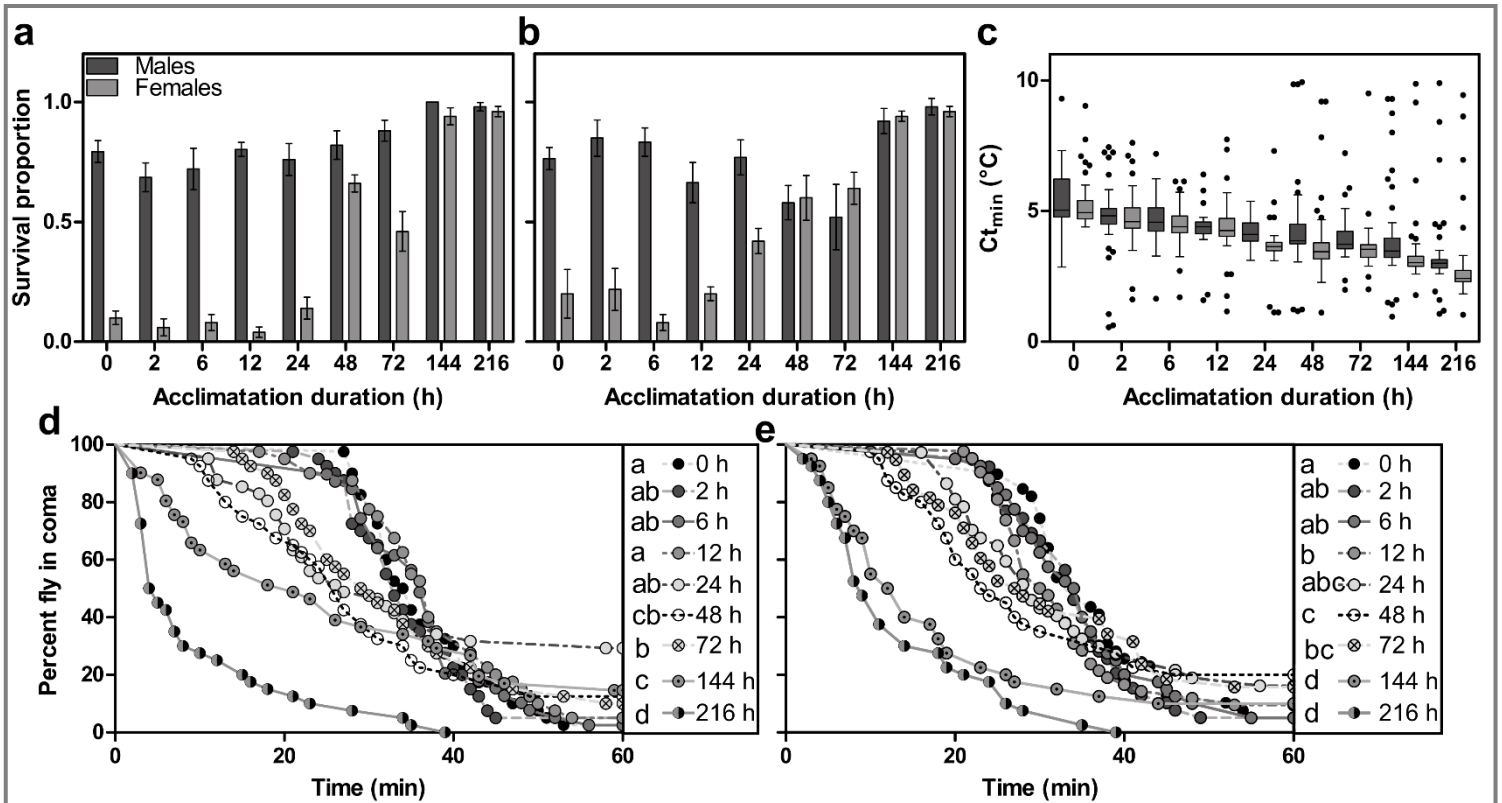


Fig. 1: Cold tolerance assays on *Drosophila suzukii* males and females. Flies were either non-acclimated or cold acclimated at 10°C for incremental durations (from 2 to 216 h). Males (dark grey) and females (light grey) survival after (a) an acute cold stress at -5°C for 60 min or after (b) a chronic cold stress at 0°C for 24 h according to cold acclimation duration. (c) Boxplots describing mean critical thermal minimum (Ct_{min}) according to cold acclimation duration for males (dark grey) and females (light grey). Chill coma recovery time (CCRT) following exposure to 0°C for 12 h according to acclimation duration (indicated in right panel) for males (d) and females (e). Groups with the same letter in the right panel are not significantly different (Gehan-Breslow-Wilcoxon test to compare survival curves, $p.value < 0.0013$).

survival increased with acclimation duration, reaching 98% for males and 96% for females after 216 h (9 days) of acclimation (acute: $\chi^2 = 278.52$, $p.value < 0.001$; chronic: $\chi^2 = 135.10$, $p.value < 0.001$; Fig. 1a and b). Overall, males showed a higher survival to acute and chronic cold stress than females (acute: $\chi^2 = 253.34$, $p.value < 0.001$; chronic: $\chi^2 = 91.509$, $p.value < 0.001$; Fig. 1a and b). Females clearly showed improved cold survival with acclimation duration, whereas benefits in males were much less manifested due to their already-high basal tolerance. These distinct patterns resulted in significant acclimation duration x gender interaction (acute: $\chi^2 = 10.75$, $p.value < 0.01$; chronic: $\chi^2 = 48.70$, $p.value < 0.001$; Fig. 1a and b).

Mean critical thermal minimum (Ct_{min}) of males and females are displayed in Fig. 1c. With no acclimation treatment (i.e. 0 h of acclimation), mean Ct_{min} values were 5.4 ± 0.15 and 5.2 ± 0.11 °C for males and females, respectively. Values gradually decreased with acclimation duration to reach 3.2 ± 0.18 and 2.7 ± 0.12 °C after 216 h acclimation, in males and females

Table 1: Comparisons of CCRT (chill coma recovery time) curves between the different acclimation durations, for males and females.

Gender / acc. duration	2 h		6 h		12 h		24 h		48 h		72 h		144 h		216 h		
	<i>p.value</i>	χ^2	<i>p.value</i>	χ^2	<i>p.value</i>	χ^2	<i>p.value</i>	χ^2	<i>p.value</i>	χ^2	<i>p.value</i>	χ^2	<i>p.value</i>	χ^2	<i>p.value</i>	χ^2	
Males	0 h	0.27	1.20	0.88	0.02	0.37	0.77	0.11	2.48	0.0004**	12.4	0.03	4.33	0.0027**	8.99	<0.0001***	60.78
	2 h	/	/	0.44	0.57	0.10	2.70	0.16	1.90	0.004*	8.29	0.13	2.20	0.0076**	7.12	<0.0001***	58.07
	6 h	/	/	/	/	0.55	0.34	0.13	2.19	0.0012	10.5	0.05	3.82	0.0048**	7.96	<0.0001***	59.91
	12 h	/	/	/	/	/	/	0.12	2.32	0.0005**	12.2	0.02	4.77	0.0022**	9.39	<0.0001***	61.14
	24 h	/	/	/	/	/	/	/	/	0.27	1.17	0.85	0.034	0.020	5.37	<0.0001***	46.00
	48 h	/	/	/	/	/	/	/	/	/	/	0.15	1.98	0.15	2.01	<0.0001***	39.84
	72 h	/	/	/	/	/	/	/	/	/	/	/	/	0.03	4.62	<0.0001***	49.98
	144 h	/	/	/	/	/	/	/	/	/	/	/	/	/	/	<0.0001***	19.69
Females	0 h	0.47	0.51	0.40	0.70	0.03	4.29	0.04	3.97	0.003*	8.38	0.04	3.88	<0.0001***	33.49	<0.0001***	55.57
	2 h	/	/	0.95	0.003	0.20	1.59	0.14	2.08	0.01	5.85	0.18	1.73	<0.0001***	30.85	<0.0001***	51.31
	6 h	/	/	/	/	0.24	1.32	0.17	1.83	0.01	5.87	0.17	1.82	<0.0001***	31.58	<0.0001***	52.56
	12 h	/	/	/	/	/	/	0.43	0.60	0.02	4.90	0.28	1.14	<0.0001***	31.00	<0.0001***	50.1
	24 h	/	/	/	/	/	/	/	/	0.13	2.24	0.58	0.29	<0.0001***	24.63	<0.0001***	38.99
	48 h	/	/	/	/	/	/	/	/	/	/	0.13	0.82	<0.0001***	15.83	<0.0001***	29.02
	72 h	/	/	/	/	/	/	/	/	/	/	/	/	<0.0001***	19.78	<0.0001***	34.51
	144 h	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0.18	1.75

CCRT curves are available on Fig. 1d and e. *p. values* has been adjusted using Bonferroni correction:
 * < 0.0013; ** < 0.0002; *** < 2.7E-05 (Gehan-Breslow-Wilcoxon tests).

Table 2: Summary of RNA sequencing results

Sample	Yield (Mbp)	N Reads	%Q30	Mean Q	N Reads after trimming	% Mapping
CAF1	8.297	33 187 088	94.98	35.76	30 158 494	71.4
CAF2	7.166	28 665 463	94.84	35.7	26 025 539	70.9
CAF3	6.782	27 126 385	94.96	35.75	24 736 334	71.5
COF1	9.01	36 040 513	95.38	35.8	32 867 578	69.7
COF2	11.272	45 089 307	95.29	35.78	41 064 935	70.2
COF3	6.922	27 686 685	95.38	35.81	25 285 497	73

Yield (Mbp): number of bases in mega bases; Q: quality score; %Q30: percentage of bases with a quality score of at least 30. CAF: cold acclimated females; COF: Control Females.

respectively ($\chi^2 = 220.95$, $p.value < 0.001$). Ct_{min} values decreased similarly with acclimation in males and females (acclimation duration x gender interaction: $\chi^2 = 2.09$, $p.value = 0.14$).

Chill coma recovery time (CCRT) curves are shown in Fig. 1d and e, for males and females respectively. All statistics comparing the CCRT curves of the different treatments (Gehan-Breslow-Wilcoxon tests) are available in Table 1. All flies from all treatments were in chill coma after 12 h at 0°C, but the recovery dynamics varied greatly with acclimation treatments. Globally, recovery time decreased with acclimation duration for both males and females, with non-acclimated flies showing the slowest recovery dynamics, and 216 h (9 days) acclimated flies the fastest.

Additional cold tolerance assays were performed to account for any physiological age distortion between control and acclimated flies. Indeed, the transcriptomic analysis were based on a comparison between control flies versus flies acclimated at 10°C. Even though aging is likely very limited at 10°C, we reasoned that physiological age of flies might be slightly different between control flies (5d-old mature flies) and acclimated flies (5d-old mature flies + 9 days at 10°C). Using the developmental zero (T0) at which metabolic activity is supposed to stop (7.2°C in *D. sukikii*, [42]), we estimated that degree days (DD) accumulated during the acclimation period (i.e., 25 DD for 9 days at 10°C) would be less than two days at 25°C (i.e., 35 DD). Therefore, we compared cold tolerance (CCRT) of two control fly sets, one of 5d-old and the other of 7d-old, with the cold tolerance of acclimated flies (5d-old + 9 days at 10°C). We confirmed that acclimated flies were much more cold tolerant than controls regardless of age (Figure S1).

RNA sequencing and qPCRs validation

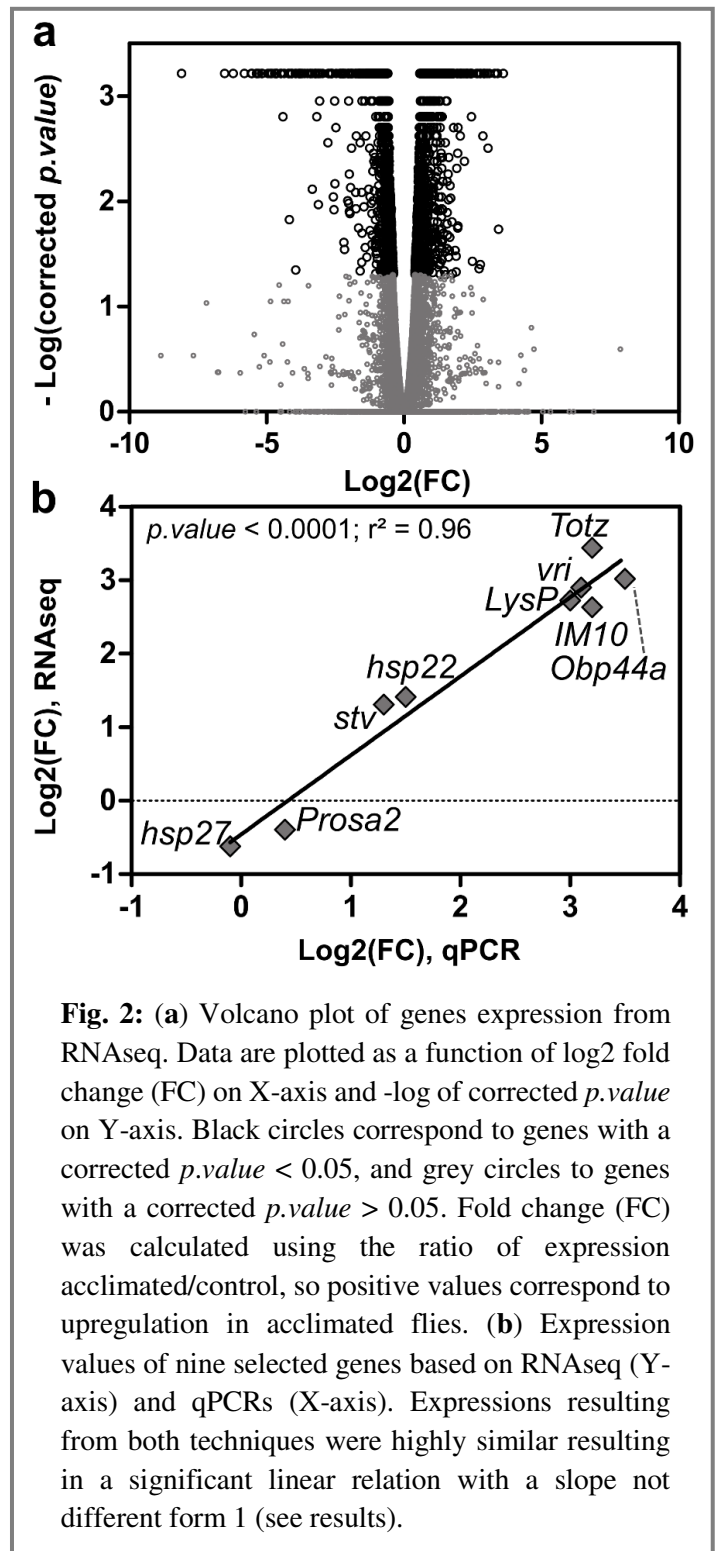
From six libraries, comprising three true replicates of control females (COF1-3) and three of cold acclimated females (CAF1-3), we obtained a total of approximately 198 million paired end reads, with an average Q30 of 95.13%. After trimming, we obtained approximately 180 million paired end reads. The mapping resulted in a mean of 71.11% mapped reads (Table 2). A total of 13486 genes were annotated and used for differential expression analysis. This analysis highlighted 1583 up- and 1325 downregulated genes in cold acclimated flies, among them 378 were expressed exclusively in cold acclimated flies, and 331 only in control flies (Fig. 2a, Tables S1 and S2). Expression patterns were validated with qPCRs on a selection of nine up- and downregulated genes (Fig. 2b) and were highly similar to expression levels resulting

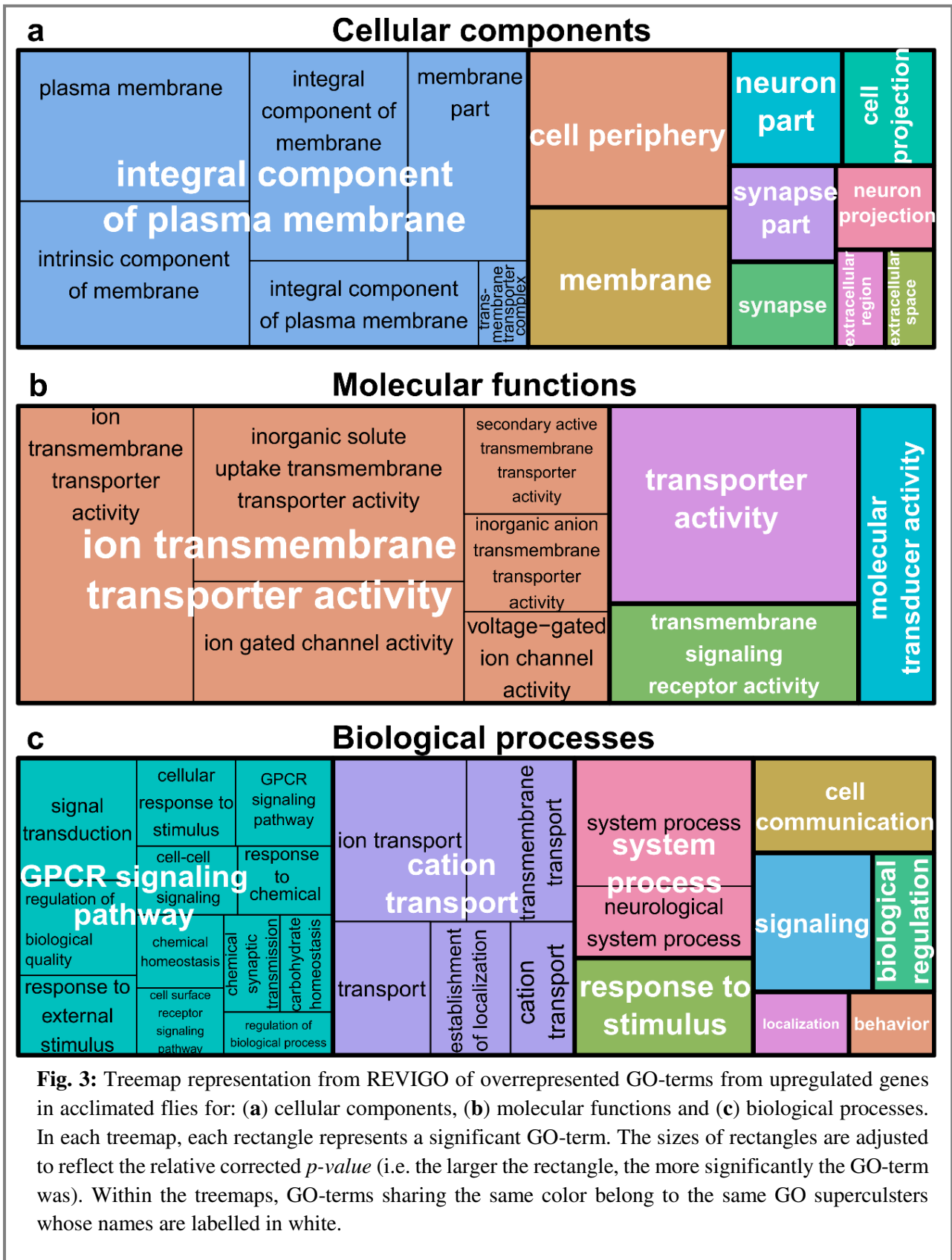
from the differential expression analysis of RNAseq (Spearman correlation: $p.value < 0.01$; linear regression: $p.value < 0.001$; $r^2 = 0.96$). The slope of this relation (0.89 ± 0.07) was not different from 1 ($F = 2.37$; $p.value = 0.14$).

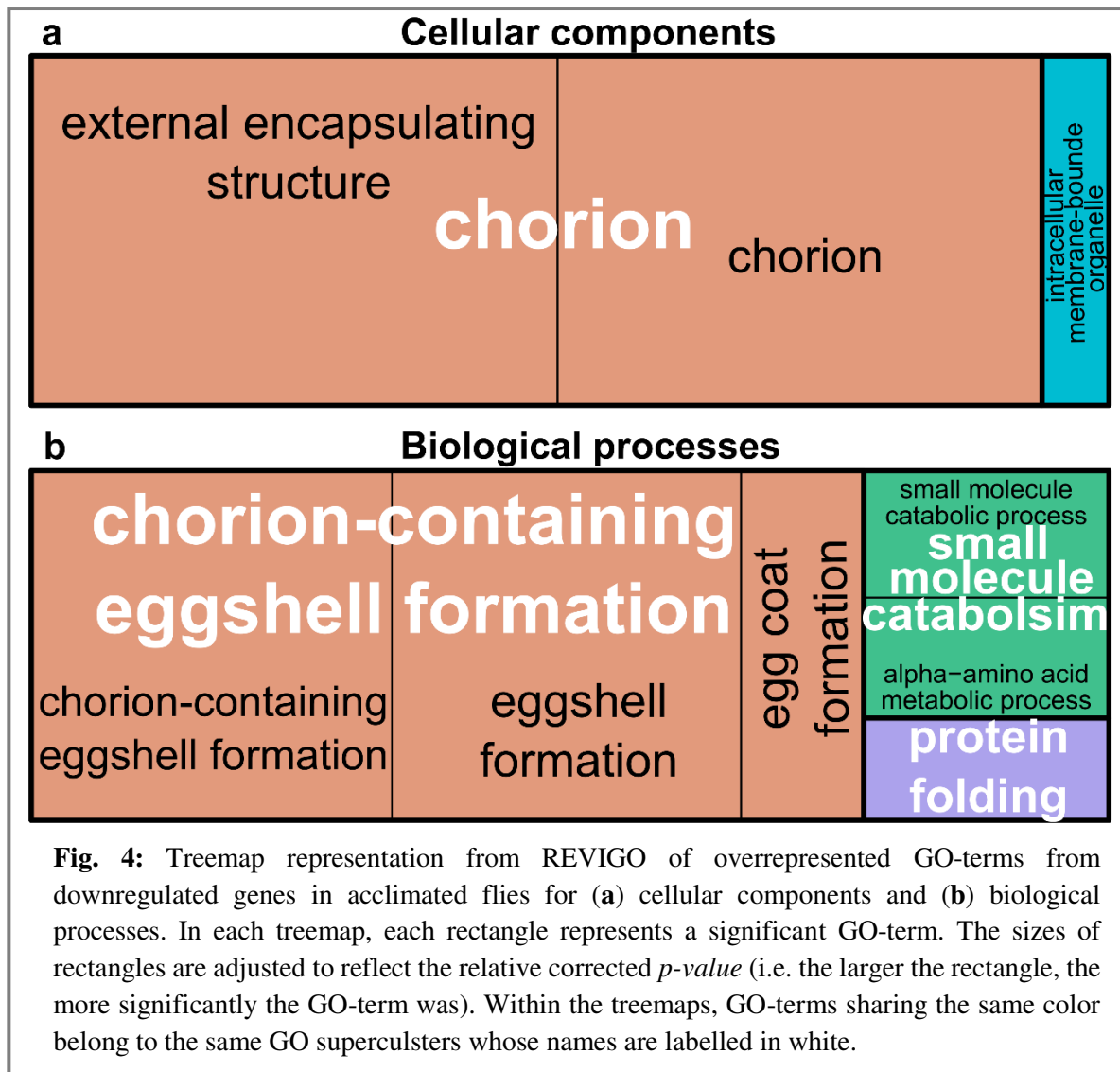
Gene ontology (GO) terms enrichment

Gene ontology terms enrichment analyses were performed separately on up- and downregulated gene sets in acclimated flies using GO-TermFinder [43]. For upregulated genes, enrichment analyses resulted in 20 significant GO-terms for cell component. These indicated that regulated genes were mainly located in ‘plasma membrane’ or ‘synapse’. Analyses also detected 26 enriched GO-terms for molecular functions, many of which were redundant and designated enrichment of ‘ion transport’ and ‘signaling across membranes’. Finally, for biological process 30 different, but sometime redundant, GO-terms were enriched. The most significant involved ‘ion transmembrane transport’, ‘response to stimulus’, ‘cell communication’, ‘signal transduction’ and various nervous system processes. ‘Carbohydrate homeostasis’ was also found to be enriched (Fig. 3, Tables S3 to S5). For the set of downregulated genes,

analyses resulted in three significant GO-terms for cell component (i.e., ‘external encapsulating structure’, ‘chorion’, and ‘intracellular membrane-bounded organelle’), no GO-term was enriched for molecular function, and 11 GO-terms were enriched for biological process, including ‘eggshell formation’, ‘vitelline membrane formation’, ‘carboxylic acid catabolic







process’ or ‘protein folding’ (Fig. 4, Tables S6 to S8). Results of these analyses were very similar to outputs obtained with DAVID annotation tool [44, 45], which detected similar enriched GO-terms. This latter analysis also found a single enriched KEGG pathway: ‘starch and sucrose metabolism’ (Tables S9 and S10).

To facilitate interpretations, functional redundancy among GO-terms was reduced, and the presence of superclusters of overrepresented GO-terms was visualized in treemaps using REVIGO program [46]. In the treemaps, representative GO clusters are shown as rectangles whose size reflects the *p*-values. Related GO-terms are then joined into superclusters that present a particular relevance. For cellular component, REVIGO found the following superclusters: ‘integral component of plasma membrane’ and ‘chorion’, for up- and

downregulated genes respectively (Fig. 3a and 4a). For molecular functions, one main GO supercluster was found for upregulated genes: ‘ion transmembrane transporter activity’ (Fig. 3b). For biological processes, three superclusters were found from upregulated genes: ‘G-protein coupled receptor (GPCR) signaling pathway’, ‘cation transport’ and ‘system process’ (Fig. 3c). GO-terms related to downregulated genes formed a single supercluster: ‘chorion-containing eggshell formation’ (Fig. 4b).

Discussion

Cold tolerance

We subjected *D. suzukii* adults to incremental acclimation durations to determine cold pre-exposure duration needed to acquire high cold tolerance. Very short acclimation durations (2 or 6 h) at mildly low temperature did not markedly improved cold tolerance (Fig. 1). In previous studies, rapid cold hardening had either no impact [37], or a positive impact [35, 36] on *D. suzukii* cold tolerance. To observe rapid cold hardening response, protocols typically involve pre-exposures to stressful temperatures (around 0°C) [47]. The temperature used in the present study to acclimate insects (10°C) was probably not low/stressful enough to trigger a rapid acquisition of cold tolerance. On the other hand, as reported in other insects [48, 49], cold tolerance increased when acclimation duration increased. We found that survival was high and maximum after six days of acclimation and then remained high with longer acclimation durations. Despite acute and chronic survival reached a plateau after six days of acclimation, CCRT and $C_{t_{min}}$ decreased further after nine days of acclimation. Our results are in accordance with previous findings showing that in *D. suzukii* acclimation at mildly low temperature for several consecutive days deeply promotes adult cold tolerance [27, 37, 40]. We therefore confirm that *D. suzukii* displays high and efficient cold tolerance plasticity; this capability likely contributes to its invasive success in temperate cold regions.

Ion transmembrane transport

GO-terms enrichment analysis revealed that the major part of upregulated genes were located in cell membranes, neurons and synapses. Molecular functions mainly involved ‘ion transmembrane transporter activity’ and many biological processes involved ‘ionic transporter activity’ (Fig. 3). Involvement of similar GO-terms has previously been reported in *D. melanogaster* flies that developed at low temperature (13°C) [50–52]. Multiple genes linked to ‘ion transmembrane transport activities’ were upregulated in cold acclimated flies (Ca^{2+} , Na^{+} or K^{+} channels: *Ca-alpha1D*, *NaCP60E*, *KCNQ*, *Hk*, *Irk2* and *Irk3* ; K^{+} transporters: *Nha2*,

Gene	Gene ID ^a	FC ^b	Function or process
<i>NaCP60E</i>	DS10_00003598	1.77	Transporters : ion channels
<i>Ca-alpha1D</i>	DS10_00000955	1.54	
<i>KCNQ</i>	DS10_00002789	1.68	
<i>Hk</i>	DS10_00004874	1.51	
<i>Irk2</i>	DS10_00011692	2.01	
<i>Irk3</i>	DS10_00006400	2.44	
<i>Vha14-2</i>	DS10_00012657	Only in CAF	ATPase ion transporters
<i>Vha68-1</i>	DS10_00007687	1.44	
<i>VhaAC39-2</i>	DS10_00009944	Only in CAF	
<i>VhaPPA1-2</i>	DS10_00011222	Only in CAF	
<i>ppk15</i>	DS10_00012645	Only in CAF	Sodium transporters
<i>ppk17</i>	DS10_00000507	2.49	
<i>ppk5</i>	DS10_00010923	Only in CAF	
<i>ppk9</i>	DS10_00003499	Only in CAF	
<i>Oatp30B</i>	DS10_00001585	1.49	Organic anion transporters
<i>Oatp33Ea</i>	DS10_00008440	2.64	
<i>Oatp33Eb</i>	DS10_00008442	1.50	
<i>Oatp58Dc</i>	DS10_00002075	2.44	
<i>Nha1</i>	DS10_00001446	1.38	Sodium:proton transporters
<i>Nha2</i>	DS10_00010553	2.23	
<i>Nhe2</i>	DS10_00001057	1.90	
<i>GABA-B-R2</i>	DS10_00012213	2.08	Neurotransmitter receptors
<i>nAChRbeta2</i>	DS10_00012765	2.56	
<i>nAChRalpha7</i>	DS10_00004780	1.57	
<i>DopEcR</i>	DS10_00010177	2.58	
<i>5-HT1B</i>	DS10_00005814	2.44	
<i>Grip</i>	DS10_00008207	1.59	
<i>GluRIIE</i>	DS10_00012665	2.45	
<i>mth</i>	DS10_00007901	Only in CAF	GPCR involved in life span and stress response
<i>mth112</i>	DS10_00003566	2.63	
<i>mth114</i>	DS10_00004634	1.71	
<i>mth115</i>	DS10_00000453	2.07	
<i>mth19</i>	DS10_00004608	1.77	
<i>Hex-tl</i>	DS10_00012733	Only in CAF	Carbohydrate metabolism
<i>Hex-C</i>	DS10_00005493	1.46	
<i>Pepck1</i>	DS10_00005716	1.58	
<i>AkhR</i>	DS10_00001466	2.56	Carbohydrate and lipid homeostasis
<i>Desat1</i>	DS10_00012265	2.07	Synthesis of unsaturated fatty acids
<i>Hsc70-2</i>	DS10_00009454	0.27	Molecular chaperones or co-chaperones
<i>Hsp60C</i>	DS10_00006901	0.68	
<i>Hsp27</i>	DS10_00003843	0.65	
<i>HIP-R</i>	DS10_00006110	0.76	
<i>Totz</i>	DS10_00013345	10.86	
<i>Hsp22</i>	DS10_00003839	2.66	
<i>Cp7Fb</i>	DS10_00006848	0.02	
<i>Cp7Fc</i>	DS10_00006849	0.04	
<i>Cp15</i>	DS10_00003769	0.04	
<i>Cp16</i>	DS10_00003771	0.01	
<i>Cp18</i>	DS10_00003768	0.00	
<i>Cp19</i>	DS10_00003770	0.03	
<i>Cp36</i>	DS10_00006850	0.09	
<i>Cp38</i>	DS10_00006851	0.09	
<i>Yp1</i>	DS10_00004890	0.13	Structural gene for the yolk (Vitellogenin)
<i>Yp2</i>	DS10_00004891	0.11	
<i>Yp3</i>	DS10_00008400	0.16	
<i>Vm26Aa</i>	DS10_00007679	0.11	Oogenesis; vitelline membrane formation
<i>Vm26Ab</i>	DS10_00008464	0.20	
<i>Vm32E</i>	DS10_00001909	0.11	
<i>Vm34Ca</i>	DS10_00001679	0.10	

Table 3, list of genes discussed in the text

^a Spotted Wing Fly Base (<http://spottedwingflybase.org/onstate.edu>).

^b Positive and negative values of fold change (FC) for upregulated and downregulated in acclimated flies respectively. CAF: cold acclimated females; COF: Control Females.

Nha1, *Nhe2* ; Na⁺ transporters: *ppk15*, *ppk17*, *ppk5*, *ppk9* ; ATPase ion transporters: *Vha14-2*, *Vha68-1*, *VhaAC39-2*, *VhaPPA1-2* ; Na⁺/H⁺ transporters: *Nha1*, *Nha2*, *Nhe2* or transmembrane organic anion transporters: *Oatp30B*, *Oatp33Ea*, *Oatp33Eb*, *Oatp58Dc*; see Table 3).

Genes linked to ion channels are intimately correlated to cold tolerance acquisition in *Gryllus pennsylvanicus* [31]. Under permissive thermal conditions, insects maintain ion homeostasis by compensating the natural leakage of ions across membranes through active transports [15, 21]. Cell membranes are highly thermosensitive macromolecules and temperature decrease induces changes of membrane fluidity, and in extreme cases, transition of membrane phospholipidic bilayer from a liquid crystalline phase to a more rigid lamellar gel phase [22, 53]. These conformation changes can in turn alter ion permeability of membranes [53], provoking loss of ions homeostasis [22, 54]. Membrane-embedded enzymes, proteins and transporters could be altered by these changes in membrane fluidity [53, 55, 56], but also by direct kinetic effects of cold temperatures [21, 33, 57], participating in the deregulation of ion balance [15, 33, 34, 58, 59]. Alteration of ion equilibrium can directly damage cells and tissues [33, 58, 60, 61] and provoke depolarization of membranes, altering action potentials of muscles and neuron cells, conducting to loss of neuromuscular functions and coma [15, 61–68]. In insects, cold tolerance acquisition is correlated with preservation of transmembrane ion balance [33, 58, 65, 69]. This has been reported in *Drosophila* flies [34, 65, 70], including *D. suzukii* [71]. One of the underlying mechanisms of ion balance preservation is likely the plasticity of ion channel thermal sensitivity and the recruitment of ion transporters [15, 21, 65, 70, 72, 73]. The maintenance of ion homeostasis allows electrochemical properties of membranes to be persevered, guaranteeing neuromuscular functions [15, 21, 65]. Our data strongly suggest that cold acclimation in *D. suzukii* induces plastic expression of genes that play essential roles in transport activity of ions across membranes, most likely to prevent disruption of ion homeostasis that may occur at low temperature [71].

Neuronal activity

Loss of neuromuscular function at low temperature may be linked to impairment of synaptic actions [21]. Indeed neurotransmitter release depends on the activity of Ca²⁺ channels, which may be impaired by depolarization [73] and altered fluidity [74] of membranes at low temperature [53]. Here we observed regulation of genes located in neurons and synapses (Fig. 3a). In addition, several genes coding for neurotransmitter receptors were regulated after cold acclimation (GABA: *GABA-B-R2*, acetylcholine: *nAChRbeta2* and *nAChRalpha7*, dopamine:

DopEcR, serotonin: *5-HT1B* or glutamate: *Grip* and *GluRIIE*, see Table 3). In cold acclimated *G. pennsylvanicus* several genes linked to neurotransmitters were also upregulated [31]. These altered gene expressions are likely related to adjustments of neurotransmitter activities at the synaptic level in order to compensate cold deleterious effects.

G-protein coupled receptor activity

The main GO-term supercluster from upregulated genes was ‘G-protein coupled receptor (GPCR) pathway’ that comprised many GO-terms related to signal transduction or signaling (Fig. 3c). ‘Response to stimulus’ and ‘cell communication’ were also highlighted among the most enriched GO-terms. This suggests a major role of genes related to cellular signaling pathways for cold tolerance acquisition. In *D. ananassae*, populations selected for cold tolerance showed upregulation of GO-terms implied in ‘cell communication’ and ‘signaling’ [75]. Interestingly, cold acclimated *G. pennsylvanicus* also showed upregulation of genes linked to ‘GPCR activity’ [31]. Among genes involved in the enriched GO-term ‘GPCR signaling pathway’, we observed several founding members the methuselah family (*mth*, *mthl12*, *mthl14*, *mthl15*, *mthl9*) that were all upregulated in acclimated insects. These genes are *Drosophila* GPCRs involved in the modulation of life span and stress response including heat, starvation, and oxidative damage [76]. GPCRs are transmembrane receptors, initiators of signal transduction and cellular responses, and are involved in a large panel of physiological functions [77]. Stress signaling and thermal plasticity in insects is regulated by protein kinases [78–80], and protein kinase signaling cascades can be activated by GPCRs [81]. Upregulated GO-terms linked to GPCR activity could therefore be linked to a global response or sensing of low temperatures, initiating transduction signal cascades triggering cold acclimation.

We identified two genes coding for G protein-coupled inwardly-rectifying potassium channels (*Irk2* and *Irk3*) that were regulated in cold acclimated flies (see Table 3). These ion channels are primary effectors of GPCR, and participate in hyperpolarization of cell membranes [82]. As previously discussed, ion channel activities are of major importance to counterbalance disturbance of ionic homeostasis due to cold temperatures. Regulation of transcripts linked to GPCR activity could therefore also be linked to ion homeostasis maintenance, through adjustment of ion channels.

Carbohydrate metabolism

We expected to observe regulation of candidate genes involved in some of the canonical cold-acclimation mechanisms, such as membrane modifications or cryoprotectant (sugar) metabolism. The GO-term ‘carbohydrate homeostasis’ was indeed enriched in acclimated flies as well as the KEGG pathway ‘starch and sucrose metabolism’. Genes upregulated in acclimated flies included several enzymes (*Hex-t1*, *Hex-C*, *Pepck1*) playing key roles in carbohydrate metabolism and sugars interconversions. We also noted the upregulation of an adipokinetic hormone receptor (*AkhR*), a GPCR neuropeptide/hormone receptor involved in carbohydrate and lipid homeostasis [83]. We also found upregulation of *desaturase* (*Desat1*), a gene well known to be involved in the synthesis of unsaturated fatty acids [84]. Desaturases play roles in cold-induced phospholipid restructuring [55] and upregulation of *Desat* genes has been correlated with enhancement of cold hardiness [85, 86]. *Desat1* and *Desat2* were also reported to be upregulated in diapausing *D. montana* females [87], but downregulation of *Desat* genes was reported in cold-acclimated *Drosophila virilis* group species [88]. Profiling with various ‘Omics’ techniques has provided supporting evidence for changes in carbohydrate metabolism and accumulation of sugars (particularly glucose, sucrose, fructose and trehalose) after both rapid and gradual cold acclimation in *Drosophila* [24, 29, 52, 89–91]. Our observation supports the general view that regulation of carbohydrate and lipid metabolism is an element of cold tolerance acquisition in *D. suzukii* [27, 39].

Stress genes

We expected to find regulation of genes involved in stress response. Functional annotation revealed ‘protein folding’ as enriched GO-term associated to downregulated genes in acclimated flies. Heat shock chaperones, such as *Hsc70-2*, *Hsp60C*, *Hsp27* or *HIP-R* (a co-chaperone), were downregulated in acclimated flies. *Hsc70-2* and *Hsp60C* are constitutively expressed and not known to be cold-responsive [92]. There is a constant need for chaperone assistance during *de novo* protein folding and refolding of polypeptide chains [93], and hence, the reduced expression of heat shock chaperones in cold acclimated flies may be linked to reduced *de novo* protein folding at mild low temperature. We found no clear indication of stress genes being upregulated in acclimated flies, except *Hsp22* (FC = 2.6) or *Totz* (FC = 10); the latter was among the most upregulated genes in cold acclimated flies (Fig. 2b, Table 3). *Totz* belongs to *Turandot* family; these genes are part of a humoral stress reaction unlike the heat shock response which mainly deals with the intracellular accumulation of denatured proteins [94]. *Turandot* genes also respond to other types of stress such as heat, UV or oxidative agents

like paraquat [94, 95]. *Turandot* genes have also been shown to respond to low temperature likely because cold activates immune pathways [95, 96].

Oogenic activity

Many genes, relatively less expressed in cold acclimated flies, were involved in ‘chorion and eggshell formation’ (forming a main GO supercluster) (Fig. 4). Genes coding for chorion proteins (*Cp7Fb*, *Cp7Fc*, *Cp15*, *Cp16*, *Cp18*, *Cp19*, *Cp36*, *Cp38*), yolk proteins (*Yp1*, *Yp2*, *Yp3*) or vitelline membrane proteins (*Vm26Aa*, *Vm26Ab*, *Vm32E*, *Vm34Ca*) were among the most downregulated genes in acclimated flies (see Table 3). In *D. melanogaster*, egg production is highest between 18 and 23 °C and is strongly reduced at temperatures below or above this range [97, 98]. Expression of chorion-related genes follows this pattern as highest expression has been reported at intermediate temperatures [50]. The lowest developmental thermal threshold for ovarian maturation is generally around 10-12°C in temperate drosophilids [99]. In *D. suzukii*, studies reported a reproductive dormancy mainly due to development at low temperature [35, 36, 39]. This reproductive arrest has also been associated with reduced expression of yolk protein gene (*Yp1*) [36]. Following the release of cold-induced dormancy, all yolk proteins transcripts have been found to be upregulated in *D. melanogaster* [100]. Reduced expression of genes related to oogenesis in acclimated *D. suzukii* females suggests a dormancy syndrome. In our experimental design, all flies developed and remained 5 days at 25°C after eclosion for maturation before treatment; therefore, all females contained mature eggs before they were cold acclimated. The gene patterns we observe here could translate a reduced oogenic activity at low temperature. Interestingly, Lirakis *et al.*, [101] provided a very detailed study of oogenesis in a range of dormancy-inducing conditions in *D. melanogaster*. They reported that one-week old mature flies (maintained at 25 °C) had active oogenesis and mature eggs. When these one-week old mature flies were transferred to dormancy-inducing conditions (10°C, 10L : 14D), mature eggs were still present but oogenesis was stopped. Hence, they demonstrated that a dormancy-like phenotype (i.e. block of oogenesis) can be observed in mature flies when exposed to low temperature. In our setting, mature *D. suzukii* flies were acclimated by transferring them from 25 to 10°C for 9 days. Just as in *D. melanogaster*, this situation could have stopped oogenic activity, explaining the relatively lower expression of genes involved in chorion and eggshell formation. It is not clear whether arrest of oogenesis is a passive consequence of low temperature with no adaptive value for cold tolerance or whether this mechanism is an active protective strategy. Data from drosophila species support that cold-induced oogenesis arrest

(via quiescence or diapause) is actually part of an integrated mechanism of cold adaptation and cold stress tolerance mechanism [99, 101].

Conclusions

This work provides a characterization of transcriptomic changes in response to cold acclimation in *D. suzukii*. Cold tolerance of *D. suzukii* gradually increased with acclimation duration leading to highly cold-hardy phenotype, adding to the body of evidence that this fly possesses high thermal plasticity. We observed major transcriptional remodeling after cold acclimation, primarily involving ion transport and various signaling pathways across membranes and within neuronal parts, and a decrease of the reproductive (oogenesis) function. We suggest these mechanisms represent the core part of the physiological strategy of cold tolerance acquisition in *D. suzukii*. This study provides a list of new candidate genes related to cold tolerance in this fly. In particular, we have highlighted regulation of many genes of interest playing putative roles in ion transport and homeostasis. These processes determine neuromuscular functions, which are highly affected by low temperature, and therefore, constitute the fundamentals of insect cold tolerance [15]. Despite that acclimation treatments used in laboratory settings are not fully ecologically relevant, it is probable that molecular mechanisms similar to those described in the present study may occur in fields during seasonal acclimatization when temperatures progressively drop in the fall.

Methods

Flies' rearing conditions and acclimation procedure

The *D. suzukii* line used in this work is a population collected from infested fruits originated from different locations in Trentino (Italia), and brought to the Vigalzano station of the Edmund Mach foundation (46.042574N, 11.135245E) in 2011. This line was sent to our laboratory (Rennes, France) in early 2016, and has been reared under lab conditions ever since. Flies were reared in glass bottles (100 mL) and supplied *ad libitum* with a carrot-based diet (for 1 liter: agar: 15 g, sucrose: 50 g, carrot powder: 50g, brewer yeast: 30 g, cornmeal: 20 g, kalmus: 8 g, Nipagin: 8 mL). Flies were kept in incubators (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan) at 25°C, 12L : 12D. Males were identified visually and were manually separated from females with an aspirator without CO₂ to avoid stress due to anesthesia [102]. Acclimation was induced as follows: 5-day old flies were held either in the rearing conditions (i.e. non-acclimated treatment) or they were cold-exposed at 10°C in incubators (Model MIR-

154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan) for 0 h, 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, 6 days (144 h) or 9 days (216 h), in order to generate nine treatments of incremental acclimation duration. Photoperiod during thermal treatments was standardized at 12L : 12D.

Cold tolerance assays

Acute cold stress

From each of the nine treatment group males and females were randomly taken and distributed in five replicates of 10 individuals that were submitted to -5°C for 60 min, using glass vials immersed in a glycol solution cooled by a cryostat (Cryostat Lauda ECO RE 630). After exposure, flies were allowed to recover in 40 mL food vials under rearing conditions. Survival was assessed by counting the number of dead and living individuals in each vial 48 h after acute cold stress.

Chronic cold stress

From each of the nine treatment group males and females were randomly taken and distributed in five replicates of 10 individuals that were submitted to 0°C for 24 h, using 40 mL food vials placed in a cooled-incubator (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan). After exposure, flies were allowed to recover in 40 mL food vials under rearing conditions. Survival was assessed by counting the number of dead and living individuals in each vial 48 h after chronic cold stress.

Critical thermal minimum ($C_{t_{min}}$)

To estimate the $C_{t_{min}}$ we used a knockdown column consisting of a vertical jacketed glass column (52 x 4.7cm) containing several cleats to help flies not falling out the column while still awake. In order to regulate the temperature, the column was linked to a cryostat (same model as for acute stress assays), and temperature was checked into the column using a thermocouple K connected to a Comark Tempscan C8600 scanning thermometer (Comark Instruments, Norwich, Norfolk, UK). The thermocouple was inserted at the center of the column, at mid height. Approximately 60 males or females of each conditions were introduced at the top of the column. Flies were allowed to equilibrate in the device at 18°C for a few minutes, after which the temperature was decreased to 0°C at 0.5°C/min. At each fly passing out and falling out of the column the $C_{t_{min}}$ (°C) was recorded. The experiment ended when temperature reached 0°C.

Chill coma recovery time (CCRT)

CCRT is defined as the resurgence time of motor activity after a cold knockdown [103]. In order to knockout flies, 40 males and females from each 9 treatments were submitted to 0°C for 12 h, using a food vial placed in a cooled-incubator (same model as chronic stress assays). Directly after exposure, flies were rapidly transferred to a 25°C regulated room, and spread on a large plane surface using a fine paint brush. Recovery time was individually recorded when a fly was able to stand up. Experimentation ended after 120 min, and non-recovered flies were counted.

Statistical analyses

Except for CCRT, all analyses of cold tolerance assays were performed using R (version 3.4.3; R Core Team, 2016). We modeled survival to -5°C and 0°C separately with generalized linear models (GLM) with logistic link function for proportion outcomes (i.e. number of dead/alive flies per vial). Ct_{min} data were analyzed using a GLM following a Gaussian error family with an identity link function. For these models, response variables were dependent on acclimation duration, gender, and the interaction between these two. We analyzed the effect of each variable through an analysis of deviance (“Anova” function in “car” package, [104]).

CCRT data were analyzed using survival analysis with the software Prism (version 5.01; GraphPad, La Jolla, CA, USA). We compared each recovery curves using pairwise comparisons (Gehan-Breslow-Wilcoxon Test) for males and females separately. Alpha level of significance for survival analyses was adjusted thanks to Bonferroni correction ($\alpha = 0.0013$).

RNA extraction and sequencing

Transcriptomic analyses were performed only on females. Three replicates of 15 females from the control group and from the 216 h (9 day) cold acclimation group were snap-frozen in liquid N₂. Samples were ground to a fine powder using pestles in 1.5 mL Eppendorf tubes immersed in liquid N₂. RNA extractions were performed using a Nucleospin Kit (Macherey Nagel, Düren, Germany) following manufacturer’s instructions. At the end of the process, total RNA was eluted in 50 μ L of RNase-free H₂O. For each sample we measured RNA concentrations using a Nanodrop 1000 (Thermo Scientific, Waltham, MA), and estimate integrity using an Agilent Bioanalyzer nanochip (Agilent, Palo Alto, CA). Bioanalyser outputs are shown in Figure S2. Next-generation RNA sequencing was performed by Eurofins Genomics (Ebersberg, Germany). RNA strand-specific libraries were created using Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina) according to the manufacturer’s instructions. Briefly, polyA-

RNA was extracted from total RNA using an oligodT-bead based method. After mRNA fragmentation, first-strand and dUTP-based second strand synthesis was carried out, followed by end-repair, A-tailing, ligation of the indexed Illumina Adapter and digestion of the dUTP-strand. Size selection was done using a bead-based method. After PCR amplification, the resulting fragments were cleaned up, pooled, quantified and used for cluster generation. For sequencing, pooled libraries were loaded on the cBot (Illumina) and cluster generation was performed following manufacturer's instructions. Paired-end sequencing using 125bp read length was performed on a HiSeq2500 machine (HiSeq Control Software 2.2.58) using HiSeq Flow Cell v4 and TruSeq SBS Kit v4. For processing of raw data RTA version 1.18.64 and CASAVA 1.8.4 were used to generate FASTQ-files. RNAseq produced six libraries: three for control and three for cold acclimated females.

Mapping, differential expression analyses and gene ontology term enrichment

All bioinformatics analyses were performed using Galaxy (<https://usegalaxy.genouest.org>). First, raw data were trimmed using Trimmomatic (version 0.36 [105]), and their quality was checked using FastQC (version 0.11.2 [106]). Reads were then mapped to *D. suzukii* reference genome [107] using Bowtie2 based Tophat (versions: 2.2.8 and 2.1.1, respectively [108]). The mapping resulted in a mean of 71.11% mapped reads. Reads were then annotated using reference annotation of *D. suzukii* [107] and assembled thanks to Cufflinks (version 2.2.1 [109]), and differential expression of transcripts was computed using Cuffdiff (Cuffdiff is part of Cufflinks, version 2.2.1 [109]). Transcript expression was considered significantly different between control and cold acclimated flies when the *q.value* (FDR-adjusted *p.value*) was < 0.05. Up and downregulated genes were extracted, converted to *D. melanogaster* orthologs, analyzed for GO-terms enrichment using Go-TermFinder [43], and results were visualized using REVIGO [46]. To ensure validity and robustness of the results, we performed a second analysis in parallel, using the functional annotation clustering of DAVID [44, 45].

qPCR

To validate RNAseq data, we performed qPCR on selected genes. List of primers are available in Table S11. For each sample, 500 ng of RNA were reverse transcribed to cDNA using Superscript III first-strand synthesis system (Invitrogen Pty, Thornton, Australia) following manufacturer's instructions. We targeted 11 genes, down- or upregulated, involved in different processes and functions (see Table S11 for details), including 2 housekeeping genes (*RP49* and *GAPDH* [110, 111]). *RP49* showed the most stable expression among the different samples,

and was then preferred over *GAPDH* as reference gene. A Roche LightCycler® 480 (Roche, Basel, Switzerland) using SybrGreen I mix (Roche) was used to perform qPCRs, following the protocol described in [92]. Relative gene expressions were calculated using the $\Delta\Delta C_t$ technique [112]. Expression level of genes resulting from qPCR were then correlated to expression levels resulting from RNAseq using Spearman non-parametric tests and a linear regression in Prism (version 5.01; GraphPad, La Jolla, CA, USA).

Data availability statement

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE126708 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126708>).

Supplementary information

Supplementary figures (.pdf):

Supplementary Figure S1: Chill coma recovery time of control flies at two different age (5 and 7 days) and flies acclimated for 9 days. Flies have been submitted to 0°C for 12 h, and then their individual time to recover from coma was recorded at 25°C. Each point corresponds to the recovery time of one fly. Full lines: females, dotted lines: males.

Supplementary Figure S2: Bioanalyser report on RNA extract from Control (COF) and cold acclimated (CAF) samples (females of *D. sukuzii*). Cold acclimation consisted of 5 days old females exposed to 10°C during 9 days. (Agilent Bioanalyzer nanochip, Agilent, Palo Alto, CA).

Supplementary tables (.xlsx):

Table S1 Differential gene expression from Cuffdiff: Upregulated genes in acclimated flies

Table S2 Differential gene expression from Cuffdiff: Down-regulated genes in acclimated flies

Table S3 Gene ontology term enrichment, Up regulated Cell component

Table S4 Gene ontology term enrichment, Up regulated Molecular function

Table S5 Gene ontology term enrichment, Up regulated Biological process

Table S6 Gene ontology term enrichment, Down regulated Cell component

Table S7 Gene ontology term enrichment, Down regulated Molecular function

Table S8 Gene ontology term enrichment, Down regulated Biological process

Table S9 Up Outcomes from DAVID annotation on up regulated genes

Table S10 Outcomes from DAVID annotation on down regulated genes

Table S11 List of primers used in qPCR

Competing interests

The authors declare there are no competing interests.

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Authors' contributions

TE and HC designed the experimental plan. TE and HC conducted all experiments. TE analysed the data and performed statistical analysis. TE and HC drafted and reviewed the manuscript.

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Discussion générale

I. Rappel du contexte de l'étude

Comprendre comment les organismes sont impactés par les conditions abiotiques de leur environnement permet de définir leur niche fondamentale (Hutchinson, 1957, 1978). Parmi les nombreuses composantes de cette niche, la température joue un rôle primordial, influant grandement sur la biologie des organismes (voir partie **I.2 de l'introduction**). La tolérance thermique d'une espèce définit donc en partie sa niche fondamentale, participant ainsi à la délimitation de son aire de répartition théorique (Sunday et al., 2012). Dans le cadre des invasions biologiques, la thermotolérance basale et induite d'une espèce influe donc sur son potentiel invasif (Bates et al., 2013; Kolar and Lodge, 2002; Zerebecki and Sorte, 2011). De plus, les conditions thermiques rencontrées par les propagules lors des phases de transport et d'introduction peuvent s'avérer délétères, compromettant leur survie. Le succès des invasions biologiques dépend de la capacité des propagules à tolérer ces stress (Renault et al., 2018). La plasticité phénotypique permet aux organismes d'ajuster leur physiologie et de tolérer une grande variété de conditions environnementales, et représente donc un facteur prédominant dans le succès des invasions biologiques (Davidson et al., 2011; Nyamukondiwa et al., 2010; Renault et al., 2018 ; voir partie **I.8 de l'introduction**).

Drosophila suzukii est une espèce invasive en Europe, en Amérique du Nord et Amérique du Sud. Cette espèce est intolérante au froid (Dalton et al., 2011; Jakobs et al., 2015), pourtant elle parvient à survivre aux températures hivernales en Europe du Nord et en Amérique du Nord. Avant de commencer cette thèse, peu d'études s'étaient intéressées à la plasticité de la thermotolérance de *D. suzukii*. De plus, les mécanismes physiologiques sous-jacents à cette plasticité n'étaient pas connus chez cette espèce. L'objectif général de ma thèse était donc d'acquérir de nouvelles connaissances fondamentales concernant la thermotolérance basale et induite de *D. suzukii* et d'identifier les mécanismes physiologiques sous-jacents à l'acclimatation thermique chez cette espèce.

Je discuterai l'ensemble de mes résultats au regard de la littérature concernant la thermobiologie de *D. suzukii* et celle d'autres insectes : dans un premier temps je m'intéresserai à la thermotolérance basale de *D. suzukii*, puis j'aborderai la plasticité de sa tolérance au froid et les mécanismes physiologiques sous-jacents à l'acclimatation. Enfin, je discuterai la portée appliquée de ma thèse dans le cadre d'un projet de lutte intégrée contre *D. suzukii*, via la technique de l'insecte stérile.

II. Thermotolérance de *Drosophila suzukii*

II.1. Thermotolérance basale

Les premiers travaux portant sur la thermotolérance de *D. suzukii* étaient relativement récents et portaient sur des souches provenant des Etats-Unis ou du Canada. La thermotolérance des souches européennes restait jusqu'à présent peu étudiée. Le premier objectif de ma thèse était par conséquent d'améliorer nos connaissances sur la thermotolérance basale de *D. suzukii*. Nous avons testé un large panel de températures froides et chaudes, avec des gradients de durées d'expositions, et pour chaque température testée, nous avons estimé les $Lt_{50}^{(1)}$, permettant de décrire une portion de courbe de performance de *D. suzukii* (Figure 14A ; voir partie **I.4 de l'introduction**).

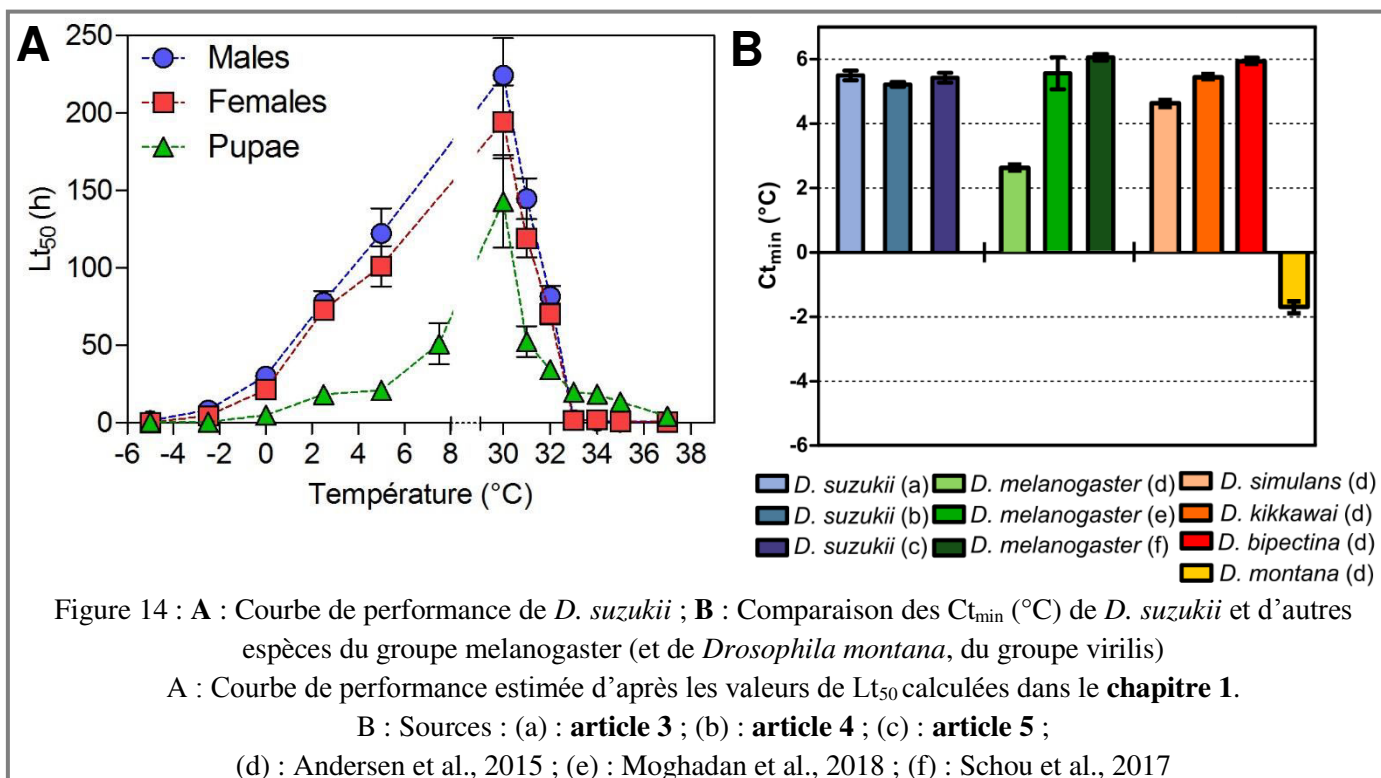
II.1.a. Tolérance au froid

Lors des expositions au froid, nous avons observé une diminution graduelle du Lt_{50} corrélée avec la diminution de la température. Chez les adultes, ces observations ont révélé un seuil entre 7,5 et 5°C, où la mortalité augmentait brutalement (20% de mortalité après un mois à 7,5°C contre 50% de mortalité après 5 jours à 5°C). Ces données (Enriquez and Colinet, 2017), qui ont depuis été confortées par de nouvelles études sur la thermotolérance de *D. suzukii* (Aly et al., 2017; Everman et al., 2018; Jakobs et al., 2017; Kim et al., 2018a), confirment qu'il s'agit bien d'une espèce typiquement intolérante au froid (Dalton et al., 2011; Jakobs et al., 2015 ; **chapitre 1**). Nous avons également observé que les pupes étaient beaucoup plus sensibles au froid que les adultes. Des études en conditions environnementales ont permis de montrer que la survie de *D. suzukii* était effectivement sévèrement réduite par les températures hivernales naturelles et que la survie des pupes était inférieure à celle des adultes (Kaçar et al., 2016; Stockton et al., 2019). Nos résultats, avec les données de la littérature, suggèrent donc que cette espèce survit aux périodes hivernales au stade adulte, ce qui est corroboré par les données de captures (Briem et al., 2018; Panel et al., 2018; Thistlewood et al., 2018). De plus, la faible tolérance au froid de *D. suzukii* explique pourquoi les populations sont réduites pendant l'hiver (Arnó et al., 2016; Mazzetto et al., 2015; Wang et al., 2016).

Les courbes de performance sont bornées, délimitées par les températures minimales, Ct_{min} , et maximales, Ct_{max}^2 , que l'espèce étudiée peut tolérer (voir partie **I.4 de l'introduction**).

¹ Lt_{50} : Temps léthal (50%) : correspond à la durée d'exposition induisant 50% de mortalité dans la population testée, à une température donnée.

² $Ct_{min/max}$: Température critique minimale/maximale. Généralement corrélée à une perte de fonction neuromotrice.



Lors de nos différentes expérimentations nous avons mesuré à plusieurs reprises la Ct_{min} basale de *D. sukuzii* (chez les drosophiles témoins des **articles 3, 4 et 5**), qui était en moyenne égale à 5,3°C (Figure 14B). Il est intéressant de noter que cette valeur de Ct_{min} correspond aux températures auxquelles nous avons observé la diminution brutale du Lt₅₀ (entre 7,5 et 5°C ; Figure 14A). Cette température semble donc représenter un seuil important dans la tolérance au froid de *D. sukuzii*. Si l'on compare les valeurs de Ct_{min} de *D. sukuzii* à celles d'autres espèces phylogénétiquement proches du groupe melanogaster, telles que *D. simulans* et *D. melanogaster* (voir Figure 10), nous pouvons observer que leur tolérance au froid est relativement similaire (Figure 14B). *D. simulans* et *D. melanogaster* sont originaires d'Afrique équatoriale (Lachaise et al., 1988) et sont maintenant cosmopolites (Keller, 2007). Malgré ces origines tropicales elles sont, comme *D. sukuzii*, capables de passer l'hiver au stade adulte dans les zones tempérées (Izquierdo, 1991). Toutefois, on observe une nette différence en comparaison à *Drosophila montana*, une espèce du groupe virilis. Cette drosophile est tolérante au froid, et est présente dans des régions de hautes latitudes et aux températures hivernales très froides (Vesala and Hoikkala, 2011).

II.1.b. Tolérance au chaud

Mes résultats représentent également un apport important concernant la tolérance au chaud de *D. sukuzii*, qui avait été peu étudiée jusqu'à présent. Les courbes de performance étant

asymétriques (voir partie **I.4 de l'introduction**), la performance diminue de manière abrupte avec l'augmentation des températures. Cette asymétrie apparaît nettement sur la courbe de performance de *D. sukukii*, (Figure 14A). En effet, l'analyse du taux de survie lors d'expositions aux hautes températures a montré un point d'inflexion entre 32 et 33°C : le Lt_{50} chutait alors de 80 à 2 heures (**chapitre 1**), dénotant une grande sensibilité aux températures élevées chez *D. sukukii*. Depuis la publication de nos résultats (Enriquez and Colinet, 2017), deux nouvelles études ont montré que la survie de *D. sukukii* était très faible lors d'expositions supérieures à 32°C (Eben et al., 2018; Evans et al., 2018), ce qui est cohérent avec nos observations. Cette faible tolérance au chaud peut expliquer les baisses de populations observées chez *D. sukukii* durant l'été (Pelton et al., 2016; Thistlewood et al., 2018). Toutefois, les pupes montraient une tolérance aux températures chaudes accrue comparativement aux adultes (un taux de survie près de 10 fois supérieur pour les températures au-dessus de 32°C). La courbe de performance des pupes montre d'ailleurs une phase décroissante plus graduelle que celle des adultes (Figure 14A). La tolérance accrue des pupes pourrait offrir à *D. sukukii* un avantage dans les régions au climat chaud, comme l'Amérique du Sud, mais également sous nos latitudes, lors des pics de chaleur estivale, sous serre par exemple. Nous avons toutefois montré que la survie des pupes était fortement impactée par un taux d'humidité relative faible. L'humidité relative est un facteur influençant grandement la biologie (G. Johnson, 1942; Mellanby, 1935) et la répartition géographique des insectes (Hoffmann, 2010; Kellermann et al., 2009), pouvant exacerber les effets d'un stress thermique (quelques exemples, chez les coléoptères : Birch, 1944 ; chez une espèce de parasitoïde : Eman, 2007 ; chez la mouche Tsétsé : Kleynhans and Terblanche, 2011 ; chez *D. melanogaster* : Bublly et al., 2012; Kobey and Montooth, 2013). Tochen et al (2016) ont observé que l'humidité relative impactait les traits de vie de *D. sukukii*, mais nos résultats sont les premiers à montrer un effet d'interaction entre la température et l'humidité relative chez cette espèce. Par la suite, l'étude de Eben et al. (2018) a conforté nos résultats en montrant qu'une faible humidité relative lors d'un stress thermique chaud impactait fortement la survie de *D. sukukii*.

II.1.c. La thermotolérance basale de *D. sukukii* permet-elle d'expliquer le succès de son invasion ?

Des études phylogénétiques ont montré que *D. sukukii* était probablement originaire de zones climatiques tempérées, telles que des forêts montagneuses (Ometto et al., 2013 ; voir **partie III.1 de l'introduction**). Cette préadaptation aux climats tempérés joue très

probablement un rôle important dans le rapide succès de son invasion. En effet, des similarités climatiques entre l'aire d'origine, et l'aire nouvellement colonisée peuvent faciliter les invasions biologiques (Renault et al., 2018). Comme nous l'avons vu précédemment, *D. suzukii* est peu tolérante au froid et sa survie est rapidement compromise lors d'expositions à des températures supérieures à 32°C (voir Figure 14). Sous nos latitudes, les températures hivernales descendent largement en dessous de 5°C (sa $C_{t_{min}}$) et durant l'été des pics de chaleurs supérieurs à 32°C sont communs, et peuvent être couplés à des faibles taux d'humidité. Par conséquent, la seule thermotolérance basale de *D. suzukii* n'explique pas qu'elle puisse tolérer les conditions abiotiques des milieux qu'elle a envahis, ce qui suggère que sa survie repose sur d'autres stratégies.

L'hétérogénéité spatiale de l'environnement des insectes offre une mosaïque de microclimats, qui peuvent fournir des conditions plus favorables lors de variations stressantes de températures (Coulson et al., 1995; Danks, 1991; Hawes et al., 2008; Pincebourde et al., 2016; Woods et al., 2015 ; voir **partie I.7.b de l'introduction**). Les drosophiles utilisent certainement des zones refuges pour survivre à l'hiver, en s'abritant sous la litière de feuilles mortes en forêt, ou dans des structures liées aux activités humaines (Dobzhansky, 1965). De même, *D. suzukii* utilise des zones refuges, telles les bordures de forêts où elle est abondamment retrouvée durant la saison hivernale, lui permettant d'éviter des températures potentiellement létales (Pelton et al., 2016; Santoiemma et al., 2018).

La plasticité phénotypique permet également aux insectes d'élargir la gamme de températures qu'ils peuvent tolérer sans effets délétères, via des processus comme l'acclimatation (voir **partie I.7 de l'introduction**), qui induisent des ajustements de leur thermotolérance de manière saisonnière (Denlinger, 1991), ou quotidienne (Kelty and Lee, 2001). En augmentant la diversité de conditions qu'une espèce peut tolérer, la plasticité phénotypique représente un atout majeur dans le succès des invasions biologiques (Davidson et al., 2011; Nyamukondiwa et al., 2010; Renault et al., 2018). Une forte plasticité phénotypique permettrait effectivement d'augmenter la thermotolérance de *D. suzukii* (Hamby et al., 2016). Par conséquent, notre second objectif était d'évaluer cette plasticité, et plus spécifiquement la plasticité de la tolérance au froid, par l'utilisation des RTF¹ et de différents régimes d'acclimatation (**chapitres 2 et 3** ; voir Figure 13).

¹ RTF : Régimes de température fluctuante

II.2. Plasticité de la tolérance au froid

II.2.a. Effets des températures fluctuantes

Dans le **chapitre 2** nous avons émis l'hypothèse que l'utilisation des RTF permettrait de réduire la mortalité au froid de *D. suzukii* en comparaison avec les températures froides constantes (TFC) (voir Figure 13). Nous avons fait varier de manière séquentielle la température, la durée et la fréquence des pics de températures des RTF. La quasi-totalité des RTF testés montraient des effets bénéfiques en réduisant la mortalité au froid des adultes et des pupes de *D. suzukii* en comparaison aux TFC, confirmant notre hypothèse. Même les durées des pics de chaleur les plus courtes (ex : 30 min), ont conduit à un abaissement de la mortalité de *D. suzukii*, ce qui montre que les mécanismes de récupération des dégâts provoqués par les températures froides se mettent en place rapidement. L'application des RTF permettait également de grandement augmenter la capacité de stockage des adultes *D. suzukii* (50% des individus toujours vivants après 130 jours). Les effets bénéfiques des RTF ont déjà été démontrés chez une grande variété d'insectes (quelques exemples : lépidoptères : Boardman et al., 2013 ; coléoptères : Lalouette et al., 2011 ; hyménoptères : Colinet et al., 2006; Foray et al., 2013; Torson et al., 2017; Yocum et al., 2011 ; diptères : Pio and Baust, 1988; Takano, 2014), y compris *D. melanogaster* (Colinet et al., 2016; Javal et al., 2016; Košťál et al., 2016b). Toutefois, nos travaux sont les premiers à permettre de tester et identifier un effet positif des RTF sur la survie de *D. suzukii* (Enriquez et al., 2018). Depuis, Grumiaux et al., (2019) ont obtenu des résultats similaires et montré que les RTF augmentaient la survie des adultes *D. suzukii* en comparaison aux TFC.

Lors de la phase de récupération des RTF, des mécanismes physiologiques sont mis en place, permettant la réparation des dégâts provoqués par les températures froides (revus dans: Colinet et al., 2018), comme le rétablissement de l'homéostasie ionique (Košťál et al., 2007), des modifications de la composition lipidique des membranes (Colinet et al., 2016), ou la production d'antioxydants (Torson et al., 2017). Les RTF induisent également des ajustements permettant d'augmenter la tolérance au froid comme lors de processus d'acclimatation, tels que l'accumulation de cryoprotecteurs et la production de protéines de choc thermique (Boardman et al., 2013; Colinet et al., 2007; Lalouette et al., 2007; Pio and Baust, 1988). Grumiaux et al (2019) ont souligné cet effet d'acclimatation procuré par les RTF chez *D. suzukii* en montrant une diminution de la CCRT¹ et de la Ct_{min} chez les drosophiles soumises aux RTF. La mise en

¹ CCRT : Chill Coma Recovery Time : durée de récupération d'un coma provoqué par une exposition au froid

place de ces mécanismes est probablement coûteuse en énergie et pourrait donc conduire à des compromis impactant la valeur adaptative des insectes, mais peu d'études se sont intéressées à cette problématique (Colinet et al., 2018).

Afin d'évaluer si les RTF peuvent induire un coût pour l'organisme testé, nous avons analysé l'impact d'expositions au froid (TFC vs RTF) au stade adulte ou pupes sur la survie des individus, la compétitivité des mâles et la fécondité des femelles. Les principales différences ont été observées chez les individus traités au stade pupes. En effet, lorsque les individus étaient soumis aux TFC au stade pupes, la survie des adultes émergeant après le stockage, la fécondité des femelles et la compétitivité des mâles étaient réduites par rapport aux témoins. Toutefois, lorsque les individus traités au stade pupes étaient soumis aux RTF, la survie était supérieure à celle des individus TFC, la compétitivité des mâles était équivalente aux témoins, mais la fécondité des femelles était plus faible que celle des femelles TFC. De ces résultats se dégagent deux conclusions :

Premièrement, le fait que la grande majorité des effets observés sur les traits de vie de *D. sukuzii* aient été identifiés chez les drosophiles issues des pupes exposées au froid (TFC ou RTF) dénote un effet de latence des dégâts liés aux températures froides (Košťál et al., 2019). Nous avons vu précédemment que les pupes de *D. sukuzii* étaient plus sensibles au froid que les adultes (**chapitre 1**). Il est donc possible que les dégâts accumulés par les pupes se répercutent par la suite au stade adulte. De tels effets latents des températures froides ont été identifiés chez d'autres insectes (exemples : pucerons : Pullin and Bale, 1988 ; lépidoptères : Marshall and Sinclair, 2015 ; diptères : Kelty et al., 1996; Yocum et al., 1994 ; *D. melanogaster* : Košťál et al., 2019). L'impact de ces effets sub-létaux sur la valeur adaptative des survivants a une forte valeur écologique (Košťál et al., 2019). En effet, dans le cadre des invasions biologiques par exemple, si les insectes survivant aux conditions de leur nouvel environnement montrent une fécondité réduite, leur potentiel invasif s'en retrouvera impacté.

Deuxièmement, les RTF parviennent à compenser les effets délétères du froid sur la mortalité après traitement, et sur la compétitivité des mâles. Toutefois, la baisse de fécondité des femelles soumises, au stade pupes, aux RTF par rapport aux femelles TFC laisse sous-entendre que les mécanismes mis en place durant les périodes de récupération des RTF ont un coût sur la valeur adaptative des femelles *D. sukuzii*, confirmant notre hypothèse de départ. Même s'il s'agit de résultats issus de variations thermiques artificielles, nos observations laissent penser que les variations thermiques journalières dans l'environnement naturel de *D.*

suzukii, conduisent à des effets complexes, et pourraient favoriser sa survie, mais aux dépens d'autres traits de vie, pouvant affecter sa valeur adaptative.

Nos résultats représentent donc un des premiers exemples montrant un compromis lié à l'utilisation des RTF chez les insectes. Les RTF ayant un fort potentiel d'utilisation pour le stockage d'insectes auxiliaires en lutte biologique (Colinet et al., 2006) ou de pollinisateurs (Rinehart et al., 2013), cet effet ne doit pas être négligé, puisque cela pourrait impacter la qualité des insectes stockés.

II.2.b. Effets de l'acclimatation

Comme chez un grand nombre d'espèces d'insectes (voir Tableau 1) l'acclimatation augmente la tolérance au froid de *D. suzukii* (acclimatation rapide : Everman et al., 2018; Toxopeus et al., 2016 ; acclimatation au stade adulte [AA] : Jakobs et al., 2015; Wallingford et al., 2016 ; acclimatation développementale [DA] : Shearer et al., 2016; Stephens et al., 2015; Toxopeus et al., 2016; Wallingford and Loeb, 2016). Nous avons testé différents régimes d'acclimatation (**Chapitre 3**) et nous nous attendions à observer une augmentation de la tolérance au froid de *D. suzukii*. La plupart des régimes d'acclimations testés abaissaient le taux de mortalité de *D. suzukii*, confirmant notre hypothèse (voir les **articles 2 à 5**). Nous avons d'ailleurs mis en évidence que lorsque la DA et la AA étaient combinées, elles avaient un effet plus important sur la tolérance au froid des drosophiles que lorsqu'elles étaient utilisées seules (**article 3**). Quelques différences sont toutefois à noter entre nos données et la littérature. Contrairement à nos observations, Jakobs et al. (2015) n'ont pas montré d'effet de l'AA sur la $C_{t_{min}}$ de *D. suzukii*, tandis qu'après DA+AA Toxopeus et al. (2016) ont observé une $C_{t_{min}}$ bien plus basse que celle que nous avons pu estimer (-1.7°C dans **l'article 4**, vs -5°C pour Toxopeus et al., 2016). Ces dissimilitudes pourraient provenir des différences d'origines entre les souches que nous avons utilisées et celle de Toxopeus et al. En effet, nos souches provenaient soit d'Italie (**articles 3 et 5**), soit de Bretagne (**article 4**), tandis que la souche utilisée par Toxopeus et al. venait d'Amérique du Nord. Ces lignées étant issues de populations provenant d'évènements d'introductions indépendants, leurs patrimoines génétiques sont sans nul doute différents. De plus les hivers étant plus rudes en Amérique du Nord, les propagules de *D. suzukii* ont pu souffrir d'une pression de sélection plus importante, favorisant les individus montrant une tolérance au froid accrue. Durant les processus d'invasions, des réponses évolutives rapides peuvent se mettre en place (Gibert et al., 2016; Goubert et al., 2017; Simberloff and Rejmánek, 2011; Urbanski et al., 2012), permettant aux espèces invasives de tolérer les conditions

abiotiques de leur nouvel environnement (Hill et al., 2013). Les drosophiles possédant un temps de génération court, elles sont capables de mettre en place des réponses évolutives rapides, comme le montre les études de sélection en laboratoire (Hoffmann et al., 2003a, 2003b), ou l'invasion de *Drosophila subobscura* en Amérique du nord et en Amérique du sud, qui est associée avec de rapides ajustements évolutifs (Balanyá et al., 2003).

Lors du transport ou de la phase d'introduction, les propagules sont soumises à des conditions stressantes et la plasticité phénotypique leur confère un avantage important, augmentant les probabilités du succès de l'invasion (Davidson et al., 2011; Nyamukondiwa et al., 2010; Renault et al., 2018 ; voir partie **I.8 de l'introduction**). La plasticité de la thermotolérance notamment, facilite l'implantation et l'expansion des espèces invasives (McCann et al., 2014; Nyamukondiwa et al., 2010; Winwood-Smith et al., 2015). Nos données (**chapitres 2 et 3**) ainsi que les données provenant de la littérature (Everman et al., 2018; Jakobs et al., 2015; Shearer et al., 2016; Stockton et al., 2018; Toxopeus et al., 2016; Wallingford et al., 2016), montrent que la tolérance au froid de *D. suzukii* est un trait fortement plastique, qui représente sans nul doute un paramètre important du succès de sa rapide invasion en Europe et en Amérique du Nord.

Les températures maximales qu'une espèce peut tolérer participent également à définir sa niche fondamentale, et influent donc sur son aire de répartition potentielle (Hoffmann, 2010; Kellermann et al., 2012b). Nos résultats concernant la tolérance basale de *D. suzukii* ont montré qu'elle était sensible aux hautes températures (< 32°C, **Chapitre 1**). Toutefois, dans la suite de nos travaux nous ne sommes pas intéressés à la plasticité de la tolérance au chaud, et à ma connaissance cela n'a pas été étudié chez cette espèce. Pourtant, la plasticité peut augmenter la tolérance au chaud des insectes (Colinet et al., 2013; Hoffmann and Hewa-Kapuge, 2000; Scott et al., 1997). Il serait par conséquent important d'étudier la plasticité potentielle de la tolérance au chaud de *D. suzukii* pour pouvoir mieux définir sa niche thermique, afin de mieux comprendre et prédire son invasion.

II.2.c. Aparté sur les « morphes d'hiver »

En plus de provoquer une augmentation de la tolérance au froid, la DA chez *D. suzukii* induit un accroissement de la taille des individus, de leur mélanisation et un arrêt de l'ovogenèse (Shearer et al., 2016; Stephens et al., 2015; Toxopeus et al., 2016). En période hivernale, les adultes *D. suzukii* capturés montrent un phénotype similaire (Guédot et al., 2018; Kanzawa, 1939; Panel et al., 2018; Shearer et al., 2016 ; voir partie **III.4.b de l'introduction**). La plupart

des études portant sur ce phénotype hivernal parlent de « morphes d'hiver », impliquant que ce phénotype serait dû à un polyphénisme, induit par les températures froides et les photopériodes faibles des périodes hivernales. J'estime cependant que ce phénotype résulte plutôt d'une norme de réaction graduelle que d'un véritable polyphénisme (voir Figure 5). En effet, une augmentation de la taille en réponse aux basses températures est commune chez les insectes (« temperature-size rule », (Atkinson, 1996), voir **partie I.3 de l'introduction**). L'augmentation de la mélanisation avec la diminution de la température a également été largement démontrée chez les insectes (Bernardo et al., 2007; Stoehr and Goux, 2008), et chez *D. melanogaster* en particulier (David et al., 1990; Gibert et al., 2000, 2007). De plus chez *D. melanogaster* la combinaison DA+AA augmente également la tolérance au froid (Colinet and Hoffmann, 2012; Slotsbo et al., 2016). Des résultats obtenus par Alexiane Kustre durant son stage de Master 2 (Kustre, 2018) suggèrent effectivement qu'il n'y a pas de véritable biphénisme chez *D. suzukii*, mais que la tolérance au froid, la taille et la mélanisation suivent une norme de réaction thermique polynomiale en fonction de la température de développement.

III. Mécanismes sous-jacents à la plasticité de la tolérance au froid chez *D. suzukii*

La plasticité de la thermotolérance des insectes repose sur des mécanismes physiologiques complexes, permettant de contrecarrer les effets négatifs des températures stressantes (voir **partie II.2 de l'introduction**). Si plusieurs études se sont intéressées à la plasticité de la tolérance au froid de *D. suzukii*, ses mécanismes sous-jacents étaient encore peu connus. L'objectif principal de ma thèse était donc d'identifier les mécanismes physiologiques liés à l'acclimatation chez *D. suzukii* en utilisant des techniques « omiques ». Au vu des ajustements physiologiques mis en place pour faire face aux températures froides chez les insectes, nous nous attendions à observer une accumulation de molécules cryoprotectrices, des modifications au niveau des GPL membranaires, des ajustements des voies métaboliques liés au métabolisme énergétique ou à d'autres fonctions impliquées dans la tolérance au froid, ainsi qu'un maintien de l'homéostasie cellulaire.

III.1. Maintien de la structure des protéines

Le froid peut impacter la physiologie des insectes de manière directe, lors d'un stress aigu (ex : -5°C / 100 min : le stress imposé aux drosophiles dans l'**article 3**), en déstabilisant la structure tridimensionnelle des protéines (Franks and Hatley, 1991; Todgham et al., 2007).

Cette dénaturation des protéines se traduit généralement par une accumulation stochastique d'acides aminés libres après le stress (Colinet et al., 2012a; Košťál et al., 2011b; Lalouette et al., 2007; Overgaard et al., 2007). Nous avons pu observer cet effet après un stress aigu chez *D. suzukii* (**article 3**). Le fait que plusieurs acides aminés essentiels se trouvaient dans le pool d'acides aminés libres suggère que cette accumulation résulterait de la dénaturation des protéines et non pas d'une augmentation de la synthèse d'acides aminés (Colinet et al., 2012a; Košťál et al., 2011b; Lalouette et al., 2007; Overgaard et al., 2007). Toutefois chez les drosophiles acclimatées (DA+AA notamment), une telle accumulation n'a pas été observée, suggérant un maintien de la structure des protéines. Ces mêmes drosophiles étaient caractérisées par une accumulation de plusieurs sucres (fructose, maltose, mannose, sucrose, et tréhalose) et acides aminés (alanine, isoleucine, leucine, phénylalanine, sérine, et valine), connus pour leurs propriétés cryoprotectrices (Kimura, 1982; Košťál et al., 2016a). Dans l'**article 4**, nous avons également identifié plusieurs sucres et acides aminés, corrélés avec l'acclimatation (DA +AA), dont certains étaient en commun avec les métabolites identifiés dans l'**article 3** : fructose pour les sucres, et leucine, phénylalanine et sérine pour les acides aminés. De plus, dans l'**article 5** nous avons observé la surexpression de plusieurs gènes impliqués dans le GO-term « homéostasie des carbohydrates ».

Les sucres, dont le fructose, sont connus comme étant liés à la tolérance au froid chez les insectes (exemples : hétéroptères : Ditrich and Košťál, 2011 ; lépidoptères Zeng et al., 2008 ; coléoptères : Fields et al., 1998 ; diptères : Baust and Edwards, 1979; Kimura, 1982), *D. melanogaster* (Colinet et al., 2012b; Košťál et al., 2011a; Overgaard et al., 2007). Chez *D. suzukii*, une étude a montré qu'après acclimatation (DA+AA) , plusieurs clusters de gènes impliqués dans le métabolisme des sucres étaient surexprimés (Shearer et al., 2016).

Les acides aminés possèdent également des qualités cryoprotectrices. L'exemple le plus marquant est celui de la proline, qui permet aux larves de *D. melanogaster* de survivre à la formation de gel extracellulaire (Košťál et al., 2012). D'autres acides aminés, tels que la Leucine, permettent d'augmenter la tolérance au froid de *D. melanogaster* lorsqu'ils sont ajoutés dans sa nourriture (Košťál et al., 2016a). De plus, l'acclimatation chez *D. melanogaster* provoque également l'accumulation de divers acides aminés, dont la leucine et la sérine (Colinet et al., 2012a).

Les cryoprotecteurs classiquement associés à la tolérance au froid des insectes sont les polyols (Denlinger and Lee Jr, 2010; Sømme, 1982; Storey and Storey, 2005). Toutefois au

cours de nos expérimentations nous n'avons pas constaté d'accumulation marquée de polyols chez les drosophiles acclimatées. Ces observations, s'ajoutant à de précédentes études, suggèrent que chez les espèces du genre *Drosophila*, la synthèse de polyols ne semble pas être liée à l'acclimatation (Kelty and Lee, 2001; Kimura, 1982; Overgaard et al., 2007).

Les similitudes entre les observations provenant de nos différentes études, ajoutées aux informations de la littérature, suggèrent que les sucres et les acides aminés (particulièrement les métabolites d'intérêt que nous avons identifiés : fructose, leucine, phénylalanine et sérine) sont importants dans le processus d'acclimatation de *D. suzukii*. Aux concentrations que nous avons mesurées (de l'ordre du nmol/mg), il est peu probable que ces molécules aient un effet colligatif (l'augmentation de la concentration en solutés entraîne une diminution de la température de congélation de l'hémolymphe, empêchant la formation de gel interne ; Storey and Storey, 1991). Elles pourraient plutôt faciliter le maintien de la structure des membranes cellulaires et des protéines (Arakawa and Timasheff, 1982; Cacula and Hinch, 2006; Carpenter and Crowe, 1988; Yancey, 2005) potentiellement par exclusion préférentielle : ces solutés interagissent préférentiellement avec les molécules d'eau, et ne se lient pas à la surface des protéines. Cette exclusion préférentielle permet donc à la protéine de rester hydratée et de garder sa conformation tridimensionnelle (Gekko and Timasheff, 1981; Kim et al., 2018b; Košťál et al., 2016a).

Le potentiel maintien de la structure des protéines que nous avons observé chez les drosophiles acclimatées (DA+AA ; **article 3**) pourrait donc être dû à l'accumulation de ces cryoprotecteurs. Toutefois nos observations sont uniquement corrélatives, et ne nous permettent pas de conclure avec certitude sur l'effet direct de l'augmentation des métabolites d'intérêt (fructose, leucine, phénylalanine et sérine) sur la tolérance au froid de *D. suzukii*. Des travaux supplémentaires sont par conséquent nécessaires, par exemple en faisant varier artificiellement les concentrations de ces différents métabolites, pour pouvoir conclure quant à leurs fonctions.

Le potentiel maintien de la structure des protéines que nous avons observé pourrait également être dû à la synthèse de PCT¹ (voir partie **II.2.a.iv de l'introduction**). Ces protéines chaperonnes sont impliquées dans le maintien de la structure des protéines et le nettoyage des protéines dénaturées, et jouent par conséquent un rôle important lors de la réponse aux stress thermiques (Burton et al., 1988; Colinet et al., 2010b; Feder, 1996). La réponse d'acclimatation

¹ PCT : protéines de choc thermique

chez *D. sukuzii* ne semble toutefois pas dépendre de la synthèse de PCT car peu d'entre elles étaient surexprimées après l'acclimatation (**article 5**). Toutefois il serait intéressant de voir si cette réponse évolue lors d'un stress aigu, et pendant la phase de récupération du stress, où les PCT sont connues pour avoir un rôle essentiel chez les insectes (exemples : hémiptères : Benoit et al., 2009 ; coléoptères : Tungjitwitayakul et al., 2015; Yocum, 2001 ; diptères : Rinehart et al., 2007) et chez *D. melanogaster* (Colinet et al., 2010a; Colinet and Hoffmann, 2012).

III.2. Adaptation homéovisqueuse des membranes

Les températures froides provoquent une rigidification des membranes cellulaires, qui peut entraîner des pertes de fonction (au niveau du transport transmembranaire notamment ; Cossins, 1994; Hazel, 1995; Košťál, 2010), induisant une perte d'homéostasie ionique, pouvant conduire au coma puis à la mort de l'individu (Hosler et al., 2000; Lee Jr, 2010; MacMillan and Sinclair, 2011a; Yocum et al., 1994). Chez les organismes tolérants au froid, l'adaptation homéovisqueuse des membranes (voir partie **II.2.a.i de l'introduction**) permet de maintenir la fluidité de la bicouche de GPL¹ et donc son bon fonctionnement (Hazel, 1995; Košťál, 2010; Lee Jr et al., 2006; Sinensky, 1974). L'adaptation homéovisqueuse se traduit généralement par des changements au niveau des têtes polaires, ainsi qu'un raccourcissement et une augmentation du taux de désaturation des chaînes carbonées des acides gras des GPL (Košťál, 2010); voir Figure 8). Nous avons donc émis l'hypothèse que l'acclimatation (DA+AA) chez *D. sukuzii* engendrerait des modifications similaires (**article 4**).

Nous avons effectivement observé des ajustements au niveau des têtes polaires des GPL tels que l'augmentation de la proportion des Lyso-GPL, des GPEtn et du ratio PE/PC¹. Ces modifications permettent d'augmenter la fluidité membranaire (voir Figure 8) et sont classiquement corrélées avec l'augmentation de la tolérance au froid chez les insectes, y compris chez *D. melanogaster* (Colinet et al., 2016; Cooper et al., 2012; Overgaard et al., 2008). Chez *D. melanogaster*, l'augmentation de la tolérance au froid est également corrélée avec un raccourcissement des chaînes carbonées (Colinet et al., 2016; Ohtsu et al., 1998, 1999; Overgaard et al., 2006). Toutefois, l'augmentation du taux d'insaturation des chaînes carbonées n'est pas toujours liée à l'augmentation de la tolérance au froid chez cette espèce (pas de modifications : Colinet et al., 2016; Cooper et al., 2012; MacMillan et al., 2009; Ohtsu et al., 1999; Overgaard et al., 2008 ; augmentation du taux d'insaturation: Cooper et al., 2014; Goto

¹ GPL : glycérophospholipides/ Lyso-GPL : lyso- glycérophospholipides/ GPEtn : phosphoéthanolamines/ GPChol : phosphocholines/ ratio PE/PC : ratio GPEtn/GPChol

et al., 2010). Dans notre étude, nous n'avons pas observé de différence au niveau de la longueur des chaînes carbonées, mais de fortes augmentations du niveau d'insaturation et de poly-insaturation. L'insaturation des acides gras est liée à l'activité d'enzymes désaturases (Cossins et al., 2002; Kayukawa et al., 2007). Ces enzymes insèrent des doubles liaisons entre les atomes de carbone, provoquant un angle d'approximativement 30° dans les chaînes carbonées, ce qui perturbe l'arrangement serré de la bicouche phospholipidique et augmente sa fluidité (voir Figure 8). Il est intéressant de noter que nous avons justement observé chez les drosophiles acclimatées (AA) l'augmentation d'un gène lié à une enzyme désaturase : *Desat1*, qui pourrait donc être lié à l'augmentation du taux d'insaturation des GPL que nous avons observée (**article 5**).

L'acclimatation chez *D. suzukii* est donc corrélée avec plusieurs signes d'adaptation homéovisqueuse, qui permettent très certainement un maintien de la fluidité et de la fonction membranaire lors d'expositions aux températures froides, prévenant la perte d'homéostasie (MacMillan et al., 2015a) et participant ainsi à prévenir les dysfonctionnements neuromusculaires (Lee Jr, 2010; MacMillan and Sinclair, 2011a).

III.3. Activité ionique des membranes et maintien de la fonction neuromusculaire

Au niveau membranaire, nous avons également observé que plusieurs GO-term¹ liés à l'activité de transporteurs ioniques étaient surexprimés après l'acclimatation chez *D. suzukii* (**article 5**). Parmi les gènes surexprimés chez les drosophiles acclimatées, nous en avons identifié plusieurs liés à l'activité de canaux ioniques (Ca²⁺, Na⁺, K⁺) et de pompes à ions (K⁺, Na⁺, Na⁺/H⁺, ATPase).

Chez les insectes, en conditions non stressantes, le déplacement passif des ions à travers les membranes est compensé par un transport actif, garantissant l'homéostasie ionique (MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). Comme présenté précédemment (voir également la partie **II.b.1 de l'introduction**), le froid perturbe la fluidité membranaire, altérant leur perméabilité aux ions (Hazel, 1995; Košťál, 2010). Les canaux et pompes à ions sont altérés par ces changements, mais aussi par les effets directs des températures froides (Cossins, 1994; Hazel, 1989; MacMillan and Sinclair, 2011a), perturbant l'équilibre entre transport passif et actif. Cette perte d'homéostasie provoque alors des cascades de dysfonctionnements

¹GO-term : Gene Ontology term, voir (The Gene Ontology project in 2008, 2008)

neuromusculaires (Andersen et al., 2015; MacMillan et al., 2014; Overgaard and MacMillan, 2017).

Les modifications d'expression des gènes des transporteurs ioniques que nous avons observées sont très probablement impliquées dans le maintien de l'équilibre ionique de la membrane et pourraient être liées à l'augmentation de la tolérance au froid provoquée par l'acclimatation. Cela a effectivement été observé chez plusieurs insectes (exemples : hémiptères : Košťál et al., 2004 ; blattoptères : Košťál et al., 2006 ; orthoptères : Coello Alvarado et al., 2015; Des Marteaux et al., 2017 ; et *D. melanogaster* : Armstrong et al., 2012) dont *D. suzukii* en réponse aux RTF (Grumiaux et al., 2019). Lors de nos expérimentations, l'acclimatation a provoqué des abaissements de la $C_{t_{min}}$ et du CCRT (**articles 3, 4 et 5**). Ces deux mesures traduisent respectivement une perte de la fonction neuromusculaire liée à une perte d'homéostasie ionique, et à un regain de l'activité neuromusculaire corrélée à un retour à l'homéostasie (MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). La diminution de la $C_{t_{min}}$ et du CCRT traduit donc une capacité à maintenir l'homéostasie à basses températures, et à rétablir l'homéostasie plus rapidement après le stress (Andersen et al., 2013; Armstrong et al., 2012; Findsen et al., 2013; MacMillan et al., 2014). Les diminutions du CCRT et des $C_{t_{min}}$ provoquées par l'acclimatation chez *D. suzukii* pourraient donc être dues à l'ajustement de l'activité des transporteurs ioniques que nous avons observée.

La perte de la fonction neuromusculaire chez les insectes pourrait être également liée à un dysfonctionnement des synapses (MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). En effet la dépolarisation des membranes induite par la perte d'homéostasie ionique et la rigidification des membranes perturbent l'activité des canaux Ca^{2+} (Findsen et al., 2016; Rohrbough and Broadie, 2005). Lors de nos travaux (**article 5**), nous avons observé la surexpression de plusieurs gènes liés à l'activité de récepteurs de plusieurs neurotransmetteurs (GABA, acétylcholine, dopamine, sérotonine, glutamate). La surexpression de ce type de gènes a déjà été montrée chez des insectes en réponse à l'acclimatation (Des Marteaux et al., 2017), et pourrait être due à des modifications de l'activité des neurotransmetteurs pour compenser les effets délétères du froid. Ces ajustements, en lien avec l'activité des transporteurs ioniques permettrait de maintenir la fonction électrochimique des synapses de *D. suzukii*, garantissant le bon fonctionnement de son système neuromusculaire (Armstrong et al., 2012; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017).

Dans le **chapitre 2** nous avons émis l'hypothèse que le faible succès reproducteur des mâles, particulièrement ceux exposés aux TFC, pouvait être en partie lié à une altération globale de leur mobilité due à l'effet des basses températures sur le système neuromusculaire. Chez les drosophiles, le succès reproducteur du mâle et le succès lors des confrontations contre ses adversaires sont directement liés à sa vitesse de course et sa vivacité (Partridge and Farquhar, 1983). Toutefois les mâles exposés aux RTF ne montraient pas de diminution du succès d'accouplement (voir également: Colinet and Hance, 2009). Il est donc envisageable que tout comme pour les drosophiles acclimatées, les drosophiles exposées aux RTF pourraient bénéficier d'ajustements de l'activité de leur transporteurs ioniques et de leurs neurotransmetteurs, garantissant ainsi leur mobilité, et leur succès lors de l'accouplement. Grumiaux et al. (2019) ont d'ailleurs montré que chez *D. sukii* les TFC provoquaient une perte de l'homéostasie, tandis que les drosophiles soumises aux RTF étaient corrélées à un maintien de l'homéostasie ionique, qui pourrait donc potentiellement faciliter le maintien de la fonction neuromusculaire.

III.4. Réserves lipidiques

Les réserves lipidiques des insectes jouent un rôle majeur dans leurs réponses aux températures froides (voir partie **II.2.C de l'introduction**). En effet, elles sont nécessaires à l'organisme pour fournir l'énergie requise à la mise en place des défenses physiologiques, des mécanismes de réparation lors de la récupération d'un stress, et permettent de tolérer le jeûne provoqué par la phase de coma lors des expositions au froid (Košťál et al., 2016b; Sinclair and Marshall, 2018).

Les TAG¹ représentent la source d'énergie principale chez les insectes (Hahn and Denlinger, 2011; Ohtsu et al., 1993; Sinclair and Marshall, 2018); voir partie **II.2.c de l'introduction**). De la même manière que pour les GPL membranaires, les températures froides entraînent une rigidification des TAG, ce qui compromet leur métabolisation. Des ajustements, comme l'augmentation du taux d'insaturation, ou le raccourcissement des chaînes carbonées permettent d'abaisser le point de fusion de ces réserves énergétiques, et d'augmenter leur fluidité aux basses températures (Košťál and Simek, 1998; Rozsypal et al., 2014; Van Dooremalen and Ellers, 2010). Nous avons observé une augmentation du degré d'insaturation des TAG chez les drosophiles acclimatées (**article 4**). Cette réponse est communément observée chez les insectes soumis aux baisses de température (Bashan and Cakmak, 2005; Hahn

¹ TAG : triacylglycerides

and Denlinger, 2011; Košťál and Simek, 1998; Van Dooremalen and Ellers, 2010), y compris chez les drosophiles (Ohtsu et al., 1993, 1999). L'augmentation de la fluidité des TAG de *D. suzukii* en réponse à l'acclimatation permet certainement de garantir la disponibilité des ressources énergétiques durant l'exposition aux températures froides, favorisant la survie de *D. suzukii* (Košťál and Simek, 1998; Rozsypal et al., 2014; Van Dooremalen and Ellers, 2010).

III.5. Dormance reproductive

L'arrêt momentané de la fonction de reproduction est une réponse aux basses températures commune chez les insectes (Košťál, 2006). L'énergie non consommée par la production de gamètes peut alors être allouée dans la mise en place des systèmes de défense ou de réparation des dégâts provoqués par les températures froides (Lirakis et al., 2018; Marshall and Sinclair, 2010; Mensch et al., 2017). Lors d'expérimentations qui ne sont pas incluses dans cette thèse, j'ai réalisé des dissections d'ovaires de femelles *D. suzukii* témoins (non acclimatées) et acclimatées (DA+AA), âgées de dix jours. J'ai pu observer que les femelles acclimatées ne

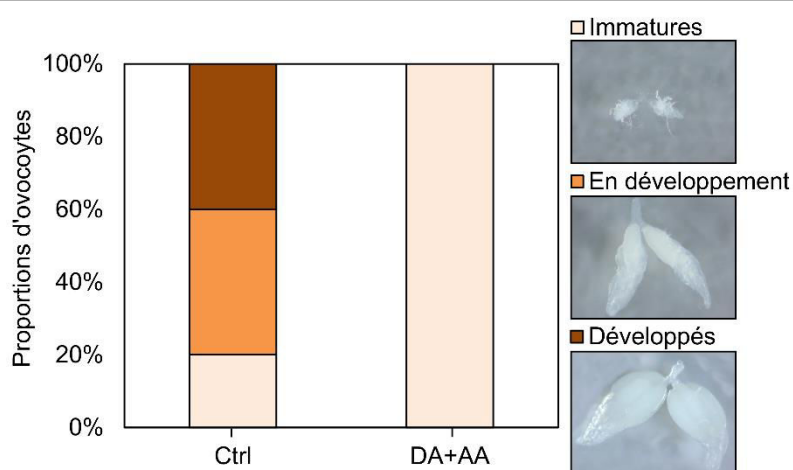


Figure 15 : Maturation des ovocytes de femelles *D. suzukii* témoins ou acclimatées.
Ctrl : Témoins ; DA+AA : combinaison de l'acclimatation développementale et au stade adulte

présentaient pas d'ovocytes matures dans leurs ovaires, contrairement aux témoins (Figure 15). De plus, nous avons observé que les drosophiles acclimatées (AA) montraient une sous-expression de plusieurs GO-term liés à la maturation des ovocytes (**article 5**). Chez *D. suzukii*, plusieurs études ont montré une dormance reproductive en lien avec un développement à basse température, notamment chez les « morphes d'hiver » (Everman et al., 2018; Shearer et al., 2016; Toxopeus et al., 2016). Cette dormance pourrait permettre à *D. suzukii* de redistribuer l'énergie normalement allouée à la reproduction vers d'autres processus physiologiques, participant ainsi à l'augmentation de sa tolérance au froid.

La valeur adaptative d'un organisme dépend à la fois de sa capacité de survie et de sa fécondité. Chez les espèces possédant un cycle de vie court, une diminution de la reproduction peut grandement affecter cette valeur adaptative (Crone, 2001). Chez les insectes, de très nombreuses études montrent des compromis entre la résistance au stress et la fécondité (ex : Chippindale et al., 1996, 1998; Huang et al., 2007; Luckinbill et al., 1984; Marshall and Sinclair, 2010; Partridge et al., 1999). La dormance représente un très bon exemple de compromis entre la survie directe et la fécondité (Lirakis et al., 2018), qui peut donc impacter la valeur adaptative future de l'organisme (Moraiti et al., 2012). La dormance semble faire partie intégrante de la stratégie de survie hivernale de *D. suzukii* (comme le confirment les données montrant un arrêt de la reproduction chez les femelles capturées durant l'hiver : Shearer et al., 2016; Wallingford et al., 2016; Zhai et al., 2016). Toutefois, les conséquences potentielles de cette période de dormance sur sa valeur adaptative dans les zones nouvellement colonisées sont encore inconnues.

III.6. Modifications de l'activité des voies métaboliques

L'acquisition de la tolérance au froid chez les insectes est liée à des ajustements importants des voies métaboliques (MacMillan et al., 2016a; Williams et al., 2014). L'un des objectifs de ma thèse était donc d'observer de tels ajustements après acclimatation chez *D. suzukii*. Les différents mécanismes mis en place pendant l'acclimatation, tels que la synthèse de cryoprotecteurs (Churchill and Storey, 1989; Storey and Storey, 1990), ou encore le maintien de l'équilibre ionique (Košťál et al., 2004), sont ATP dépendants, et donc coûteux en énergie. Nous nous attendions donc à observer des modifications au niveau du métabolisme énergétique en réponse à l'acclimatation.

Les différents processus d'acclimatation que nous avons testés chez *D. suzukii* résultaient en des métabotypes (la composition métabolique résultant d'un phénotype) clairement distincts (**articles 3 et 4**). Nous avons observé chez les drosophiles acclimatées (DA+AA) une concentration en métabolites intermédiaires du cycle de Krebs plus importante que chez les drosophiles témoins (**article 3**). Dans nos analyses suivantes, nous avons observé l'effet de l'acclimatation sur les voies métaboliques (**article 4**) et sur l'expression des gènes de nos drosophiles (**article 5**). Cependant, nous n'avons pas observé de modifications au niveau du cycle de Krebs ou de la glycolyse (**article 5**). Toutefois, pendant la phase de récupération d'un stress aigu (**article 3**), les drosophiles acclimatées (DA+AA) montraient une diminution de la concentration des sucres et des intermédiaires du cycle de Krebs. La réparation des dégâts

provoqués par les températures froides et le rétablissement de l'homéostasie étant énergétiquement coûteux, cette diminution pourrait être due à une mobilisation du métabolisme énergétique pendant la phase de récupération (Williams et al., 2016).

Les drosophiles acclimatées étaient également caractérisées par l'accumulation de différents cryoprotecteurs (voir partie **III.1 de cette discussion**) et l'ajustement de plusieurs voies métaboliques, dont notamment le métabolisme des purines et la biosynthèse des aminoacyl-ARNt. Comme mentionné dans la discussion de l'**article 3**, étant donné que ces deux processus sont essentiels au fonctionnement de l'organisme, ils sont impliqués dans une large gamme de fonctions (Ibba and Söll, 2000; Kamatani et al., 2014), il est donc difficile d'inférer quant à leur rôle potentiel dans l'augmentation de la tolérance au froid. Il est également possible que la modification de l'activité de ces voies métaboliques résulte de changements non adaptatifs, non liés à la tolérance au froid, mais provienne d'une altération globale du métabolisme provoquée par les températures froides (Colinet et al., 2012a; Overgaard et al., 2007; Teets et al., 2012; Williams et al., 2014).

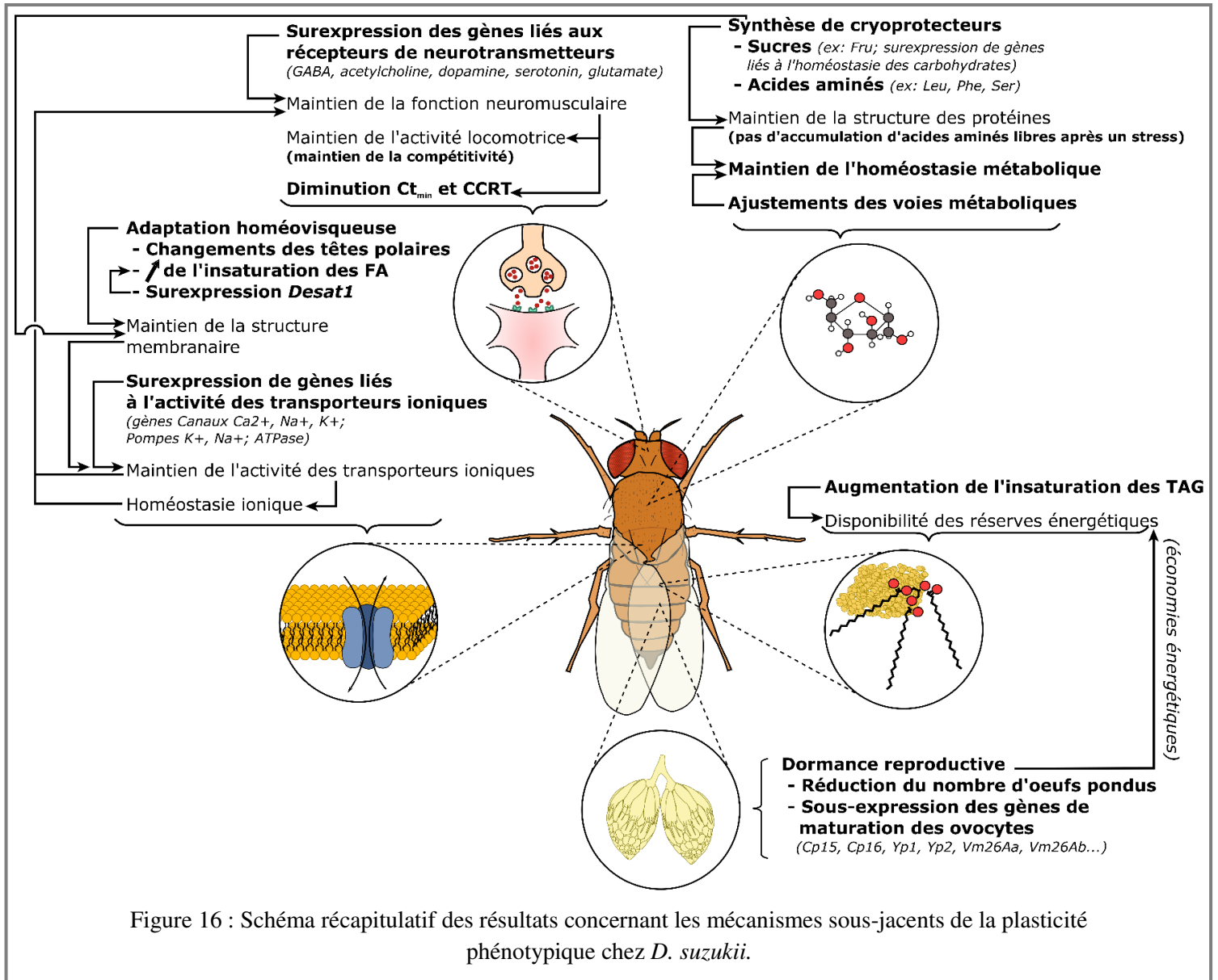
III.7. Maintenance de l'homéostasie

Les températures froides provoquent un ralentissement de la cinétique de certaines réactions enzymatiques (Košťál et al., 2004; MacMillan and Sinclair, 2011a; Zachariassen et al., 2004) et la dénaturation de certaines protéines (Franks and Hatley, 1991; Todgham et al., 2007 ; voir partie **III.1 de cette discussion**). Ces altérations sont probablement à l'origine des dérégulations globales des voies métaboliques (Košťál et al., 2016b; Michaud et al., 2008; Michaud and Denlinger, 2007; Overgaard et al., 2007; Williams et al., 2014), pouvant entraîner l'accumulation de déchets toxiques et de dérivés réactifs de l'oxygène (Rojas and Leopold, 1996; Štětina et al., 2018). L'ensemble de ces perturbations se traduit par une perte d'homéostasie du métabolisme de l'organisme (Colinet et al., 2012a; Overgaard et al., 2007; Teets et al., 2012; Williams et al., 2014). L'acclimatation chez les insectes permet d'ajuster l'activité des enzymes (Burnell et al., 1991; Joannis and Storey, 1996) et provoque des ajustements aidant au maintien de la structure des protéines (voir partie **III.1 de cette discussion**). Ces modifications garantissent le bon fonctionnement de l'organisme à basse température. Par conséquent, l'augmentation de la tolérance au froid provoquée par l'acclimatation est corrélée avec un maintien de l'homéostasie métabolique (Colinet et al., 2012a; Overgaard et al., 2007; Williams et al., 2014).

Nous avons donc émis l'hypothèse que l'acclimatation chez *D. suzukii* permettrait un équilibre des fonctions vitales au froid, qui serait caractérisé par un maintien de l'homéostasie (**article 3**). Nous avons pu observer qu'après un stress froid aigu, les drosophiles non acclimatées étaient effectivement caractérisées par une perte d'homéostasie métabolique, symptomatique d'une dérégulation causée par les températures froides (Colinet et al., 2012a; Overgaard et al., 2007; Teets et al., 2012; Williams et al., 2014 ; **article 3**). Toutefois, les drosophiles les plus tolérantes au froid (DA+AA) montraient un maintien de l'homéostasie, confirmant que ce phénomène est intimement lié à l'augmentation de la tolérance au froid chez les insectes (Colinet et al., 2012a; Overgaard et al., 2007; Williams et al., 2014).

Nos différents travaux ont permis d'identifier un grand nombre de modifications physiologiques liées à l'acclimatation chez *D. suzukii*, telles que l'accumulation de cryoprotecteurs, un maintien de la fluidité membranaire, une surexpression des gènes liés à différents transporteurs ioniques, des ajustements de l'activité de plusieurs voies métaboliques ainsi que des modifications de la composition des réserves lipidiques. Ces ajustements (résumés dans la Figure 16), participent très probablement au maintien des différentes fonctions biologiques de *D. suzukii* lors des expositions aux basses températures, ce qui explique que l'homéostasie soit maintenue chez les drosophiles acclimatées après un stress aigu.

Ces ajustements, qui sont communs à d'autres espèces d'insectes issus des milieux tempérés, sont très probablement liés à l'augmentation de la tolérance au froid de *D. suzukii*, jouant un rôle important dans sa survie hivernale et donc dans le succès de son invasion. Toutefois, nos résultats ne nous permettent pas de conclure sur la véritable valeur écologique de ces modifications. En effet, nos travaux ont eu lieu en conditions de laboratoire et il serait donc nécessaire de mettre en place des expérimentations afin de vérifier si de tels mécanismes sont mis en place par *D. suzukii* en conditions naturelles.



IV. L'utilisation du froid en lutte biologique : Projet Suzukill

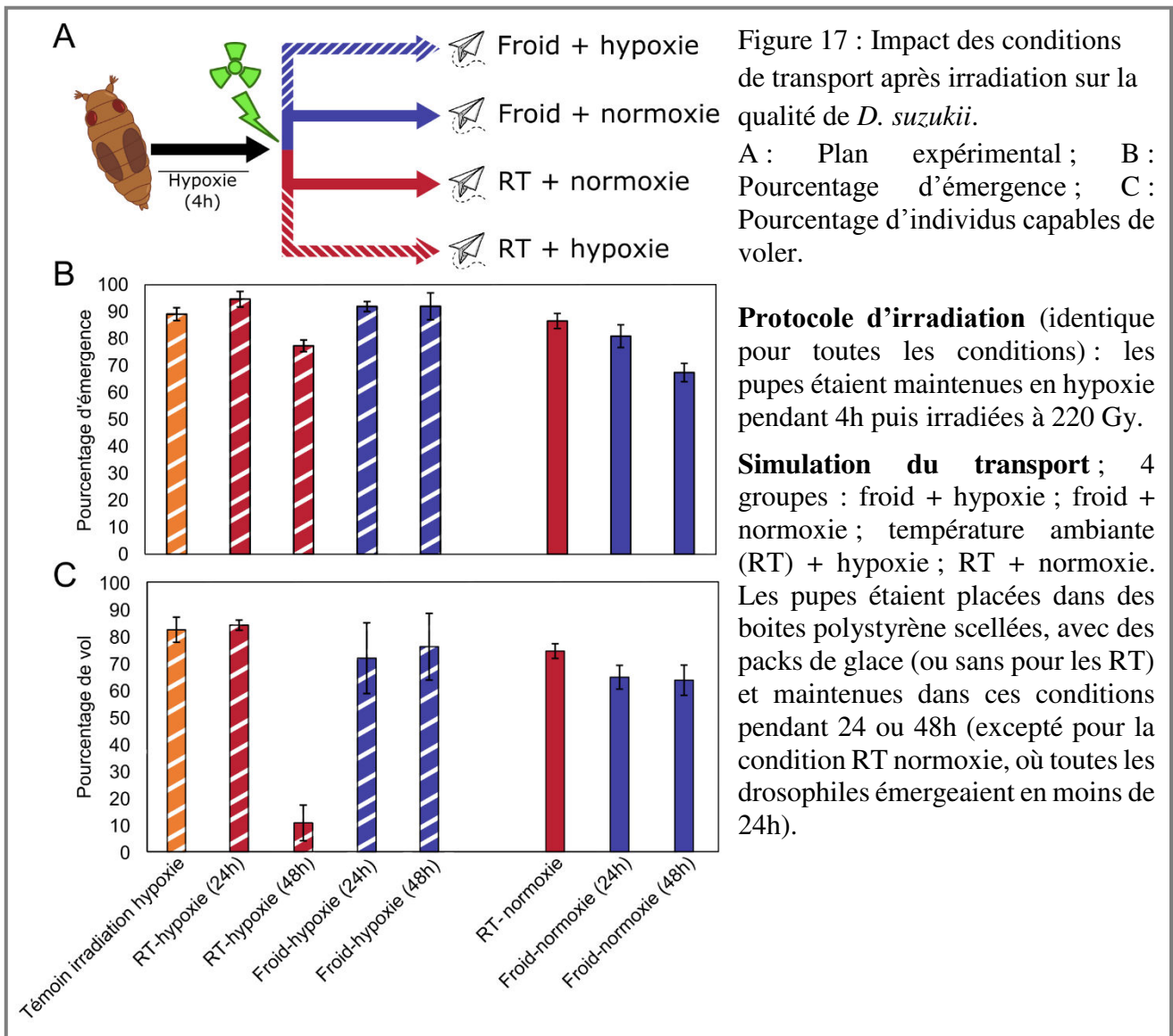
Drosophila sukukii s'attaque aux fruits mûrs, à des périodes proches de la récolte. Par conséquent les traitements pesticides sont difficiles à mettre en place contre cette espèce sans que les fruits récoltés contiennent des doses importantes de pesticides, nuisibles à la santé des consommateurs et à l'environnement. Par conséquent, plusieurs travaux sont en cours pour mettre en place des protocoles de lutte biologique contre *D. sukukii*. C'est dans ce contexte que vient s'intégrer le projet ANR Suzukill (projet collaboratif franco-autrichien) qui vise à développer la technique dite de l'insecte stérile (TIS) pour contrôler les populations de *D.*

suzukii sous serre (voir **annexe 1**). Le principe de la technique est de produire en masse des mâles *D. suzukii*, de les stériliser par irradiation puis de réaliser des lâchers sur les zones ciblées. Les insectes stériles vont inonder la population du ravageur et s'accoupler avec les individus sauvages. Le zygote issu de l'accouplement meurt alors dès les premières étapes de l'embryogenèse (Dyck et al., 2005). En répétant l'introduction de mâles stériles, la TIS peut réduire les populations sous un seuil acceptable ou dans certains cas aboutir à une éradication locale de l'espèce ciblée (Knipling, 1979).

La mise en place d'une telle technique nécessite une production de masse d'insectes et requiert l'utilisation des températures froides lors de plusieurs moments clefs (Dyck et al., 2005; Sørensen et al., 2012). En effet, des temps de latence peuvent avoir lieu entre la production des insectes et leur utilisation sur le terrain. Il est alors nécessaire de mettre en place des protocoles de stockage à basse température pour pouvoir conserver les insectes en attendant qu'ils soient utilisés sur le terrain. Comme nous l'avons vu au cours de cette thèse, *D. suzukii* est peu tolérante au froid. Des expositions prolongées au froid pourraient induire des dommages qui compromettraient la performance des insectes stockés, et donc le succès du protocole de lutte. Il serait par conséquent envisageable de mettre au point des stratégies pour augmenter la tolérance au froid de *D. suzukii* en prévision de ces périodes de stockage. Les RTF par exemple, permettent à *D. suzukii* de supporter de longues périodes d'exposition et semblent donc être une option prometteuse pour le stockage à court et moyen termes (plusieurs jours pour les pupes et plusieurs semaines pour les adultes ; voir **chapitre 2**). L'acclimatation augmente également la tolérance au froid de *D. suzukii* et pourrait être utilisée en prévision du stockage. De plus, les cryoprotecteurs que nous avons identifiés lors de nos travaux pourraient être utilisés en tant que suppléments alimentaires afin d'augmenter artificiellement la tolérance au froid de *D. suzukii*.

Le transport des individus stériles des sites de production jusqu'aux sites de lâchers représente également une phase critique. Pour des raisons pratiques les individus sont généralement transportés au stade pupes. Les conditions de transport doivent donc être adaptées afin de protéger les pupes et d'empêcher l'émergence, afin de garantir la qualité des insectes qui seront lâchés sur le terrain. Afin de prévenir l'émergence des pupes, le transport est souvent réalisé en condition d'hypoxie et à basse température (Dyck et al., 2005). Toutefois, nous avons mis en évidence que les pupes de *D. suzukii* sont particulièrement sensibles au froid (**chapitres 1 et 2**) et l'hypoxie représente également un stress physiologique important. Nous avons donc

mis en place une série d'expérimentations pour tester l'impact de ces conditions sur la qualité des pupes de *D. sukuzii* (Figure 17).



Nous avons irradié des pupes puis simulé quatre conditions de transport (le protocole est présenté plus en détail dans la Figure 17A et dans la légende de la figure). Les pupes étaient ensuite replacées en conditions de laboratoire, et nous avons mesuré leur taux d'émergence ainsi que la capacité des émergents à voler. L'hypoxie, le froid, ou la combinaison des deux n'avaient pas d'effet sur le taux d'émergence des pupes après la phase de transport (Figure 17B). Toutefois, la capacité de vol des émergents était grandement diminuée après un transport de 48h en hypoxie seule. Cet effet délétère n'était plus observé lorsque l'hypoxie était combinée aux températures froides (Figure 17C). L'hypoxie est une condition très stressante, induisant notamment une accumulation de DRO, qui provoquent des dégâts physiologiques importants.

Ces derniers pourraient ainsi être à l'origine de la baisse du taux de vol. Nous pouvons toutefois supposer que lorsque les pupes sont soumises aux températures froides, leur activité métabolique est réduite, permettant de diminuer le taux d'accumulation des DRO (Boardman et al., 2016), ce qui pourrait ainsi expliquer le maintien de la fonction de vol des drosophiles.

Que ce soit au niveau du stockage des insectes, ou au niveau des conditions de transport, l'utilisation des températures froides est un paramètre important à contrôler pour garantir la qualité des insectes stériles. Comme nous avons pu le voir, nos résultats peuvent être utiles lors de ces différentes étapes et sont donc à prendre en compte dans le développement de la TIS contre *D. suzukii*.

V. Conclusions et perspectives

Mes différents travaux ont permis d'acquérir de nombreuses connaissances fondamentales sur la thermotolérance basale et induite de *D. suzukii*. J'ai pu confirmer que cette espèce était intolérante au froid (**chapitre 1**), mais que sa thermotolérance était très plastique (**chapitres 2 et 3**). La plasticité phénotypique est une composante importante dans le succès des invasions biologiques (Davidson et al., 2011; Nyamukondiwa et al., 2010; Renault et al., 2018 ; voir **partie I.8 de l'introduction**). La plasticité de la tolérance au froid de *D. suzukii* a donc probablement conféré un avantage aux propagules, leur permettant de mieux tolérer les conditions abiotiques lors des phases de transport et d'introduction dans les nouveaux milieux, qui sont des filtres écologiques importants (Renault et al., 2018).

Mes travaux ont également permis d'identifier un grand nombre de mécanismes physiologiques sous-jacents à l'acclimatation, qui étaient encore peu connus chez cette espèce, tels que la synthèse de cryoprotecteurs, des modifications de la composition des phospholipides membranaires, un ajustement de l'expression des gènes des transporteurs ioniques, une augmentation de la fluidité des réserves lipidiques et des modifications de l'activité de plusieurs voies métaboliques. Ces ajustements participent probablement à augmenter sa tolérance au froid, expliquant le maintien de l'homéostasie après un stress aigu observé chez les drosophiles acclimatées. Ces résultats montrent que *D. suzukii* est similaire aux autres espèces de drosophiles de milieux tempérés, telles que l'espèce cosmopolite *D. melanogaster*. En effet, leur thermotolérance est semblable, et les mécanismes sous-jacents à l'acclimatation que j'ai identifiés chez *D. suzukii* sont analogues chez *D. melanogaster*. Toutefois, mes conclusions sur les effets potentiels de ces ajustements physiologiques sur la tolérance au froid se basaient

uniquement sur des corrélations et n'ont donc pas été démontrées expérimentalement chez *D. suzukii*. Il est en effet possible que les modifications métaboliques ou les différences d'expression de gènes observés soient justes des effets provoqués par les expositions aux basses températures, mais qu'ils n'aient pas de liens avec la tolérance au froid. Il serait donc nécessaire de tester l'effet des métabolites (fructose, leucine, sérine, phénylalanine) et des gènes (*Desat1*, gènes des canaux et pompes à ions) d'intérêt que j'ai identifiés, sur la tolérance au froid de *D. suzukii*. Concernant les métabolites, des expériences de suppléments alimentaires pourraient être mises en place (Košťál et al., 2016a), et pour tester la fonction précise des gènes, des techniques de « silencing » pourraient être utilisées (Colinet et al., 2010a).

Lors de mes expérimentations, j'ai pu mettre en évidence que lorsque les pupes étaient exposées à basse température, des effets sub-létaux se répercutaient sur les traits d'histoire de vie des émergents. Ces effets sub-létaux, en affectant la fécondité des femelles ou la compétitivité des mâles, pourraient avoir un impact direct sur la valeur adaptative de *D. suzukii*. Les captures en périodes hivernales indiquent une dormance reproductive chez cette espèce (Shearer et al., 2016; Wallingford et al., 2016; Zhai et al., 2016). La dormance peut également induire des compromis, influant sur la valeur adaptative des insectes (Moraiti et al., 2012). De tels compromis pourraient impacter la pérennité des populations de *D. suzukii* dans les zones envahies. Par conséquent, des expérimentations supplémentaires seraient nécessaires afin de tester si cette période de dormance affecte effectivement sa valeur adaptative.

Mes résultats représentent également un apport important concernant la tolérance au chaud de *D. suzukii*, qui n'avait été que peu décrite jusqu'à présent. Cette espèce semble peu tolérante au chaud, ce qui expliquerait les baisses de populations en période estivale (Pelton et al., 2016; Thistlewood et al., 2018). Toutefois, je n'ai pas étudié la plasticité de la tolérance au chaud chez cette espèce. Les températures maximales qu'une espèce peut supporter délimitant son aire de répartition (Hoffmann, 2010; Kellermann et al., 2012b), il serait par conséquent essentiel d'évaluer la plasticité de la tolérance au chaud chez *D. suzukii*. Ces données permettraient de mieux comprendre le succès de son invasion dans les zones de climat chaud, les variations de population durant l'été et l'impact potentiel que le réchauffement climatique pourrait avoir sur cette espèce.

Les résultats que j'ai obtenus ne reflètent peut-être pas la réalité écologique de la thermobiologie de *D. suzukii*. En effet, lors de mes expérimentations j'ai utilisé des rampes de variations thermique ne correspondant pas forcément aux variations de température que *D.*

suzukii peut rencontrer dans son environnement. Il serait par conséquent intéressant de réaliser des études sur des drosophiles provenant du terrain, à différentes périodes de l'année, pour voir si les modifications provoquées par l'acclimatation que j'ai identifiées en laboratoire ont effectivement une valeur écologique.

En plus de la modulation de la thermotolérance, la survie hivernale de *D. suzukii* repose certainement sur d'autres stratégies. En effet, dans leur étude Vega et Corley (2019) montrent qu'en intégrant les données de $C_{t_{min}}$ de *D. suzukii* dans leurs modèles, les prédictions ne sont pas suffisantes, puisque des données de terrain attestent de la présence de *D. suzukii* dans des régions où les modèles ne prédisent pas son établissement. Ces divergences entre prédictions et réalité du terrain indiquent bien que sa survie dépend d'autres stratégies, comme l'utilisation de microclimats tamponnés (Santoiemma et al., 2018; Thistlewood et al., 2018; Tonina et al., 2016; Wallingford et al., 2018; Zerulla et al., 2015).

La thermotolérance fait partie des paramètres délimitant la niche fondamentale d'une espèce et influe donc sur sa capacité d'invasion (Bates et al., 2013; Kolar and Lodge, 2002; Zerebecki and Sorte, 2011 ; voir **partie I.2 de l'introduction**). Bien que mes résultats nécessitent d'être confirmés par des données issues du terrain, ils représentent un apport important concernant la thermobiologie de *D. suzukii*. Avec les données de la littérature (ex: Dalton et al., 2011; Evans et al., 2018; Stockton et al., 2018) ces résultats permettront de délimiter les limites physiologiques de *D. suzukii* et donc de nourrir les modèles prédictifs visant à extrapoler sa niche fondamentale, afin de mieux comprendre l'évolution de son aire d'invasion, sa phénologie dans les zones envahies, ainsi que les variations démographiques d'une saison à l'autre (Gutierrez et al., 2016; Langille et al., 2016, 2017; Ørsted and Ørsted, 2019; Santos et al., 2017; Vega and Corley, 2019). Nos données montrent également un grand intérêt pour la mise en place de protocoles de lutte intégrée contre *D. suzukii*, qui couplées à ces prédictions permettront de lutter efficacement contre ce ravageur.

Une préadaptation aux milieux tempérés (Ometto et al., 2013), une tolérance au froid similaire à l'espèce cosmopolite *D. melanogaster* (voir Figure 14B), une forte plasticité de sa thermotolérance (Hamby et al., 2016 ; voir **partie II.2 de cette discussion**) ainsi que l'utilisation de microclimats (Santoiemma et al., 2018; Thistlewood et al., 2018; Wallingford et al., 2018) sont autant de facteurs délimitant la niche fondamentale de *D. suzukii*, et expliquant sa survie hivernale sous nos latitudes. A ces éléments viennent s'ajouter une forte capacité de dispersion (Tait et al., 2018; Wong et al., 2018), un cycle de vie court (Mitsui et al., 2010;

Tochen et al., 2014), une fécondité importante (Emiljanowicz et al., 2014; Kanzawa, 1939), une large gamme d'hôtes (Kenis et al., 2016; Poyet et al., 2015) une faible compétition avec d'autres espèces de drosophiles (Kanzawa, 1939; Lee et al., 2011), une faible pression de prédation (Cancino et al., 2015; Poyet et al., 2013; Rossi Stacconi et al., 2013). Ces éléments sont autant d'avantages permettant d'expliquer le succès de sa rapide invasion en Europe et sur le continent Américain.

Mes résultats offrent également un apport non négligeable concernant les connaissances fondamentales en thermobiologie, puisque mes observations ont permis de compléter la littérature portant sur les mécanismes physiologiques de la tolérance au froid chez les insectes, par exemple en montrant une nouvelle fois que la tolérance au froid est intimement liée avec l'homéostasie métabolique chez les insectes (Colinet et al., 2012a; Overgaard et al., 2007; Williams et al., 2014), et en identifiant pour la première fois des coûts liés à l'utilisation de régimes de température fluctuante, pouvant impacter la valeur adaptative des insectes.

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Annexes

Annexe 1

Sterile insect technique and *Wolbachia* symbiosis as potential tools for the control of the invasive species *Drosophila suzukii*

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Sterile insect technique and *Wolbachia* symbiosis as potential tools for the control of the invasive species *Drosophila suzukii*

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Abstract

Drosophila suzukii, a vinegar fly originated from Southeast Asia, has recently invaded western countries, and it has been recognized as an important threat of a wide variety of several commercial soft fruits. This review summarizes the current information about the biology and dispersal of *D. suzukii* and discusses the current status and prospects of control methods for the management of this pest. We highlight current knowledge and ongoing research on innovative environmental-friendly control methods with emphasis on the sterile insect technique (SIT) and the incompatible insect technique (IIT). SIT has been successfully used for the containment, suppression or even eradication of populations of insect pests. IIT has been proposed as a stand-alone tool or in conjunction with SIT for insect pest control. The principles of SIT and IIT are reviewed, and the potential value of each approach in the management of *D. suzukii* is analyzed. We thoroughly address the challenges of SIT and IIT, and we propose the use of SIT as a component of an area-wide integrated pest management approach to suppress *D. suzukii* populations. As a contingency plan, we suggest a promising alternative avenue through the combination of these two techniques, SIT/IIT, which has been developed and is currently being tested in open-field trials against *Aedes* mosquito populations. All the potential limiting factors that may render these methods ineffective, as well as the requirements that need to be fulfilled before their application, are discussed.

Keywords Biological control · Greenhouse · Incompatible insect technique · Integrated pest management · Spotted wing *Drosophila*

Key message

- *Drosophila suzukii* has invaded the Americas and Europe, and it has become a significant global pest of a wide variety of soft fruit crops.
- We review current knowledge on management practices used so far to control *D. suzukii* and discuss innovative biological control methods.
- The SIT can be used as part of an area-wide integrated pest management (AW-IPM) strategy to control *D. suzukii* in greenhouses and other confined locations.
- A combined SIT/IIT strategy should be considered as a contingency plan.

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Introduction

Invasive species have been recognized as important threats of biodiversity and cause substantial yield and revenue losses in agricultural systems (Bolda et al. 2010; Goodhue et al. 2011; Pimentel et al. 2000, 2005). The spotted wing *Drosophila* (SWD), *Drosophila suzukii*, was originally described by Matsumura in Japan in 1931. Recently, *D. suzukii* has invaded North and South America (Bolda et al. 2010; Deprá et al. 2014) and Europe (Calabria et al. 2012; Cini et al. 2012). The most probable source for the western North American populations seems to be southeast China and Hawaii, while European populations are probably originated from northeast China, with evidence of limited gene flow from eastern USA as well (Fraimout et al. 2017). Members of the *Drosophila* genus are not generally considered as pests since their larvae are primarily developed on damaged or rotting fruits. Nevertheless, *D. suzukii* infests healthy ripening fruits while still on the plant. *Drosophila suzukii* larvae consume the fruit pulp inside the fruits rendering them unmarketable and decreasing the processed fruit quality (Walsh et al. 2011). Moreover, the wounds created on the infested fruits during oviposition provide access to secondary fungal or bacterial infections leading to additional fruit tissue collapse (Asplen et al. 2015; Cini et al. 2012; Goodhue et al. 2011; Ioriatti et al. 2015; EPPO 2010; Walsh et al. 2011). *Drosophila suzukii* has a wide range of hosts including both cultivated fruits and wild plants (Asplen et al. 2015;

Diepenbrock and Burrack 2017; Grant and Sial 2016; Poyet et al. 2014, 2015). This species has thus become a significant worldwide pest (Fig. 1) of a large variety of commercial fruit crops. *Drosophila suzukii* is differentiated from other drosophilids based on two key morphological traits: (a) females have an enlarged serrated ovipositor which enables them to infest and cause physical damage to the ripening fruit and (b) males are characterized by a dark spot on the leading edge of the wings.

Significant damage has been observed in several commercial soft fruits, such as blackberries, blueberries, cherries, raspberries, strawberries, tomatoes, grapes, cherries, figs, kiwis (Ioriatti et al. 2015; Lee et al. 2015; Poyet et al. 2015; EPPO 2010; Rota-Stabelli et al. 2013; Tochen et al. 2014). Recent studies have shown that specific host fruits favor the oviposition and development of larvae, while temperature plays a crucial role on *D. suzukii* development, survival and fecundity (Ioriatti et al. 2015; Lee et al. 2011a, b; Tochen et al. 2014).

First records of *D. suzukii* in North America date back to 2008 (Hauser and Gaimari 2009; Walsh et al. 2011). In Europe, this fly was first recorded in Spain in autumn 2008 (Calabria et al. 2012) and in North Italy in 2009 (Grassi et al. 2011). By the end of 2010, *D. suzukii* had colonized the Western and Eastern USA, Canada, and most of the Mediterranean regions (Rota-Stabelli et al. 2013). Latest records report the presence of this pest in additional countries such as Austria, UK, Belgium, Germany, Hungary, Romania Turkey, Ukraine, Brazil, Chile, Argentina and Uruguay (Asplen et al. 2015; Calabria et al. 2012; Cini et al. 2012; Chireceanu et al.

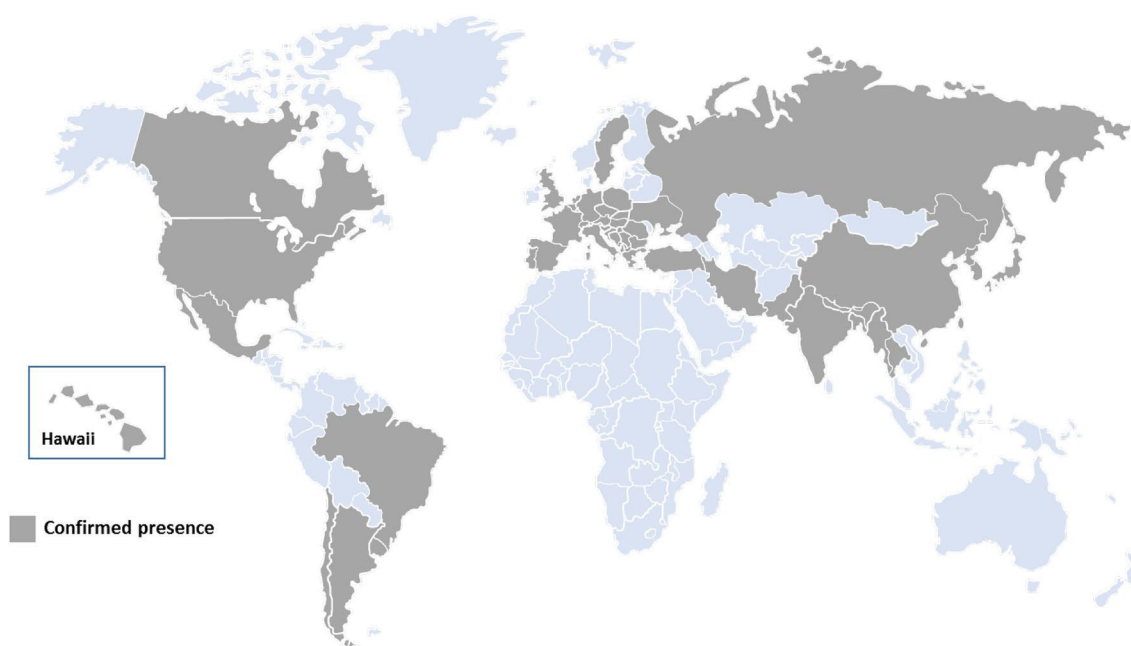


Fig. 1 Worldwide confirmed distribution of *D. suzukii* (as of August 2017)

2015; Deprá et al. 2014; Lavrinienko et al. 2017; Lengyel et al. 2015; Lue et al. 2017; Orhan et al. 2016; Servicio Agrícola y Ganadero (SAG) 2017; Vilela and Mori 2014). The expansion of *D. suzukii* in North and South America and in Europe has been very fast and widespread (Adrion et al. 2014; Lasa and Tadeo 2015). Adults of *D. suzukii* demonstrate a high dispersal potential, which is mainly attributed to the increasing global trade and the pest's invasion behavior (Calabria et al. 2012; Lengyel et al. 2015; Rota-Stabelli et al. 2013). From an ecological standpoint, *D. suzukii* adapts easily to environments with high humidity and moderate temperatures (EPPO 2010; Ometto et al. 2013). These environments allow the pest to overwinter when fruit resources are not available and low temperatures are not optimal for fermentation and fly activity (Ometto et al. 2013; Rota-Stabelli et al. 2013). Absence of natural predators and/or effective parasitoids against *D. suzukii*, as well as competitors for fresh fruits (Chabert et al. 2012; Rota-Stabelli et al. 2013), facilitates its establishment in the invaded habitats. Finally, *D. suzukii* shows a high reproductive rate and rapid developmental rate which results in 7–15 generations per year, depending on the weather conditions (Tochen et al. 2014).

Drosophila suzukii has caused substantial yield and revenue losses in agricultural systems. In the USA (California, Oregon and Washington), losses were estimated around \$511.3 million annually at 20% damage of strawberries, blueberries, raspberries, blackberries and cherries in 2008 (Bolda et al. 2010; Goodhue et al. 2011; Walsh et al. 2011). Increased costs owing to monitoring and management programs of the pest could also decrease revenue. Regulatory restrictions applied on shipments from infested areas (e.g., quarantine) could lead to significant economic impact. Residual pesticide levels exceeding tolerated limits in fruits from infested areas or postharvest treatments may also lead to rejection of exported fruits, thus limiting fruit market exploitation (Goodhue et al. 2011; Walsh et al. 2011). As a result, global economic loss for fruit production areas is potentially huge.

Considering the significant and rapidly growing agricultural costs generated by the worldwide invasion of *D. suzukii*, we review the knowledge gained so far about the tools that have been deployed to combat this pest. Following a brief review of the current management practices, the classical biological control procedures, we discuss innovative biological control methods and their potential as management solutions for facing the challenge posed by *D. suzukii*.

Pest management: current state and perspectives

Drosophila suzukii has become a key economic pest, and therefore the development of efficient monitoring and management tools is deemed indispensable. Understanding the

pest's invasion mechanisms and gaining higher resolution on its biology are needed to improve management practices (Bahder et al. 2015; Lee et al. 2011a, b). The diverse array of alternate host fruits used by *D. suzukii* and its extreme polyphagy behavior contribute to its persistence in distinct geographic areas, thus escalating its effective management into a challenge (Diepenbrock and Burrack 2017; Lee et al. 2011a, b). Currently several control methods, such as the classical chemical control, are applied worldwide to manage this pest. Nevertheless, these methods proved to be either non-effective or non-cost-effective or with limited applicability due to regulatory restrictions. In the following sections, we address biological and innovative pest management approaches and discuss their application potential and drawbacks.

Biological control

Given legitimate concerns over the risks and limitations of using a chemical control method, research efforts have already been focused on the development of environmentally sound and sustainable methods. There is a wide variety of biocontrol agents including fungi, bacteria, viruses and natural enemies of the pest that could be employed in the control programs for *D. suzukii*.

Natural enemies

Natural enemies of insect pests are endemic species that occur abundantly in agricultural fields. Natural enemies including pathogens, predators and parasitoids can be specialists or generalists, and they can induce a high level of mortality in their hosts (Flint and Dreistadt 1998). Biological control approaches based on arthropod natural enemies are currently studied and developed worldwide.

Parasitoid species are insects attacking other arthropods in the egg, larval or pupal developmental stages. They develop inside, or on the surface of the egg, larvae or pupae, and consume the host tissues during their development (Godfray 1994). Various *Drosophila* species are subjected to strong selective pressures by egg, larval and pupal parasitoids which play a key role in their population suppression. Most studies agree that *Drosophila* parasitoids induce a high rate of mortality on their host populations although the level of parasitism varies with breeding sites, local conditions and seasons (Fleury et al. 2009). Studies on natural parasitoid enemies of *D. suzukii* in its invaded regions have shown that parasitism rates are limited, and thus their use is non-efficient for population suppression (Chabert et al. 2012; Daane et al. 2016; Miller et al. 2015). This is attributed to the fact that *D. suzukii* exhibits a high level of resistance to the majority of the larval parasitoids tested, associated to a highly efficient cellular immune system (Poyet et al. 2013;

Kacsoh and Schlenke 2012). However, a recent study by Rossi Stacconi et al. (2015) showed that Italian populations of *Leptopilina heterotoma* (Hymenoptera: Figitidae) were able to overcome the encapsulation process by *D. suzukii* under standard laboratory conditions, a fact that could be attributed to the high virulence level of the wild parasitoid population collected in Italy (Rossi Stacconi et al. 2015). In contrast, generalist pupal parasitoids (e.g., *Pachycrepoides vindemiae* and *Trichopria c.f. drosophilae*) were able to develop on *D. suzukii*, at least under laboratory conditions (Chabert et al. 2012; Daane et al. 2016; Miller et al. 2015; Rossi Stacconi et al. 2015; Wang et al. 2016). Kacsoh and Schlenke (2012) used a diverse panel of parasitoid wasps and found that *D. suzukii* was able to survive infection due to the production of a constitutively high hemocyte level. High hemocyte loads are involved in encapsulation of parasitoid eggs and enable *D. suzukii* larvae to produce a vigorous immune response (Kacsoh and Schlenke 2012). Moreover, spontaneous parasitization of *D. suzukii* by *P. vindemiae* has been recently reported suggesting a gradual adaptation of the local fauna to the new invader (Rossi-Stacconi et al. 2013). In field-sampling studies in Japan, three larval endoparasitoids were reported to develop on *D. suzukii*, *Asobara japonica* (Hymenoptera: Braconidae), *G. xanthopoda* and *Leptopilina japonica japonica* (Hymenoptera: Figitidae) (Daane et al. 2016; Kasuya et al. 2013; Mitsui et al. 2007). Ongoing studies on biological control of *D. suzukii* by parasitoids are now focusing on the description of the efficiency of parasitoid species in the native area.

Progressively, government regulations require the development of host-specialized biological control agents. Consequently, a diverse array of other natural enemies including predators, entomopathogenic fungi and nematodes which are commercially available was evaluated for their ability to reduce *D. suzukii* adults and larvae survival (Woltz et al. 2015; De Ro 2016). Our current knowledge suggests that *D. suzukii* suppression by these enemies was insufficient due to low predation and infection rates, low residual activity or decreased efficiency in field trials. Taken together, extensive field studies and detailed evaluations are required to identify a novel strategy based on introduction and establishment of natural enemies of *D. suzukii* from its native range for a long-term control and determine their effectiveness and safety with regard to nontarget species.

Innovative biological control methods

Sterile insect technique (SIT)

The sterile insect technique (SIT) is a species-specific and environment-friendly method of pest population suppression or eradication (Fig. 2a). The SIT relies on repetitive

releases of mass-produced sterile insects (Dyck et al. 2005). The method is based on the sterilization of males (although releases of both sterile males and females have been successfully used), mainly using ionizing radiation which causes dominant lethal mutations in the sperm. In brief, the SIT comprises the following steps: (a) the target species is mass reared, (b) males are separated, when feasible, and sterilized and (c) released in the target area. A sufficient number of sterile males to create an overflow ratio over a period of time are released, and they are expected to compete with wild males and mate with wild females (Dyck et al. 2005). Mating results in infertile eggs and the developing zygotes die during early embryogenesis, thus inducing sterility in the wild females. Therefore, over time, the target population declines or it is potentially eradicated (Knipling 1979).

Effectiveness of SIT is undoubtedly associated with the ability of irradiated males to compete with wild males for mating with wild females. The competitiveness of released sterile males might be impacted by the insect strain and the rearing method, radiation sterilization, marking, stress during cold storage, shipment to the release site and release procedure (Dyck et al. 2005). Therefore, it is essential that the impact of the domestication, irradiation dose, as well as all other components of the SIT package, on emergence rate, adult longevity and mating competitiveness is checked and assessed prior to field application (Dyck et al. 2005; Zhang et al. 2016). Performance of sterile males is not the only critical factor that could affect success of SIT. In any SIT program, the number of released sterile males must surpass the number of wild males in the release area to compensate any negative effect associated with domestication, mass rearing, storage and their overall handling so that they mate with wild females allowing introduction of sufficient sterility into the wild population (Barnes et al. 2015; Dyck et al. 2005; Vreysen 2006). The same is true for any population suppression program, no matter if it is based on classical genetic, transgenic or symbiont-based approaches.

Apart from being an environmentally sound biological control approach, the SIT can be easily integrated with other biological control strategies (parasitoids, predators and pathogens). It is a species-specific method, and the release can be performed from the air thus overcoming any topography limitations. Successful development and application of an SIT operational program depends on: (a) the target population being at low levels; (b) extensive knowledge on the genetics, biology and ecology of the target pest being available before the application; (c) mass-rearing facilities being available and capable of providing large numbers of high-quality sterile insects; (d) a release technology having been developed, and the sterile individuals being efficiently monitored; (e) the releases being applied on an area-wide basis covering the whole pest population and (f) the released

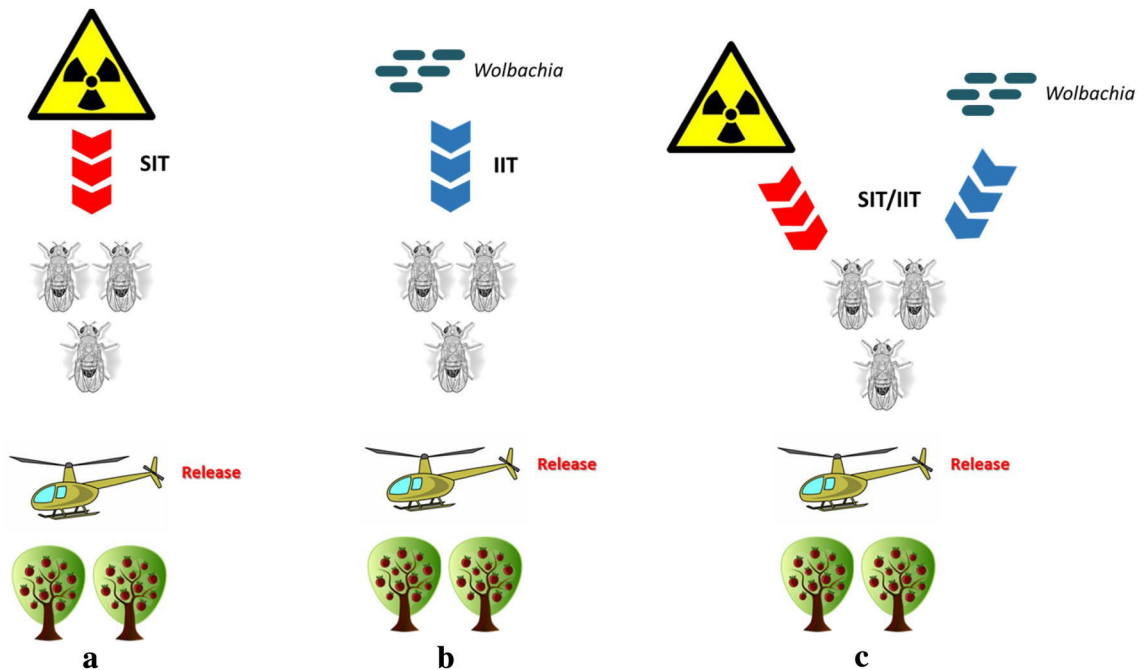


Fig. 2 **a** Sterile insect technique (SIT). Males are sterilized by the application of irradiation, **b** the incompatible insect technique (IIT). Males are sterilized by *Wolbachia* trans(infection), **c** combination of

SIT and IIT. Male sterility is due to both irradiation and *Wolbachia* infection. In all three cases (**a–c**), males are released in the field to sterilize the wild females of the targeted population

sterile individuals not causing any side effects on humans or the environment (Barnes et al. 2015; Vreysen 2006).

The SIT has proven to be a powerful control tool when applied as part of an area-wide integrated pest management (AW-IPM) approach for the creation of pest-free areas or areas of low pest prevalence (Vreysen 2006). The use of the SIT was initially put into practice in the USA, and it was subsequently developed and applied worldwide by the Joint Food and Agriculture Organization of the United Nations/International Atomic Energy Agency (FAO/IAEA) Programme on Nuclear Techniques in Food and Agriculture and collaborators (Barnes et al. 2015; Bourtzis and Robinson 2006; Dyck et al. 2005; Vreysen 2006).

The SIT has been refined over many decades, and renewed interest has recently emerged to use it for the population control of human diseases vectors (Bourtzis et al. 2016). Several successful applications of the method have been reported worldwide including the control of key insect pests such as the screwworm fly, the tsetse flies, fruit flies, Lepidoptera (moths) and disease vectors of livestock and humans (Barnes et al. 2015; Bourtzis et al. 2016; Calla et al. 2014; Lees et al. 2015; Munhenga et al. 2016; Pereira et al. 2013; Vreysen et al. 2014; Zhang et al. 2015a). The majority of the SIT programs have been applied for the control of fruit fly species as they represent one of the major insect groups of economic importance (FAO/IAEA 2013, <https://nucleus.iaea.org/sites/naipc/dirsit/>; Pereira et al. 2013).

The acknowledged deliverables of these applications have encouraged researchers to focus on ways to improve the performance of mass-reared sterile males as well as the handling and release methods. Significant knowledge acquired from SIT applications on the genera *Anastrepha*, *Bactrocera* and *Ceratitidis* can be transferred partly or entirely to other insect pest species control programs (Pereira et al. 2013).

The rapid dispersal of *D. suzukii* and its subsequent impacts on crops encourage the development of a biocontrol method with a SIT component. Radiation biology experiments are currently ongoing on *D. suzukii*, and first results have shown that X-ray radiation can inhibit the development of all stages (egg, larva, pupa and adult) of *D. suzukii* and induce adult sterility (Follett et al. 2014; Kim et al. 2016). Nevertheless, there are some reasonable concerns about the feasibility of SIT for this pest considering its high fecundity and the recurrent immigration of flies into the crop that are not completely confined. The short generation time of *D. suzukii* indicates that SIT management should be intensive, otherwise there is a risk that the population will recover rapidly. In addition, control of large field populations of *D. suzukii* poses an extra challenge for SIT. In our opinion, greenhouses and other confined locations seem to provide an ideal environment for the biocontrol of *D. suzukii* by using the SIT. The exclusion netting high tunnels could be promising candidates for the implementation of SIT. Recent studies on plastic- and mesh-covered tunnels have shown that

D. sukukii populations are significantly decreased in these confined areas, not only due to their physical exclusion, but also because of the unfavorable microclimate that is created in these locations (Rogers et al. 2016). Although complete exclusion is not achievable solely by this technique, its combination with SIT could increase the biocontrol levels of *D. sukukii*, thus limiting the use of insecticides. An additional challenge is that an adequate sexing system is not available for *D. sukukii*, and this means that both males and females will be included in the mass-reared and released flies. Bisexual SIT has been successfully used in the past; however, male only releases have been shown to be by far more cost-effective and efficient (Rendon et al. 2000).

Incompatible insect technique (IIT)

Wolbachia is a widespread endosymbiont of arthropods and filarial nematodes. *Wolbachia* can act as both a parasite and a mutualist, but it is best known for its ability to manipulate their host reproduction (for a review see Werren 1997). Four distinct reproductive alterations have been described in arthropods: feminization, parthenogenesis, male killing and cytoplasmic incompatibility (Saridaki and Bourtzis 2010; Werren et al. 2008). Collectively, these phenotypes are commonly referred to as “reproductive parasitism,” and they increase the frequency of infected females in the host population either by inducing a female-biased sex-ratio in the offspring of infected females, or by reducing the female production by uninfected females (Engelstädter and Hurst 2009).

Among the reproductive abnormalities associated with *Wolbachia* infections, cytoplasmic incompatibility (CI) is the most prominent one. *Wolbachia* induces modification of the paternal nuclear material which results in failure of progeny to develop unless the same *Wolbachia* strain(s) is/are present in the egg and exert(s) the respective rescue function(s) (Bourtzis et al. 1998; Werren 1997; Zabalou et al. 2008). *Wolbachia*'s ability to manipulate the host reproductive system along with its great pandemic has largely been recognized as potential environmental-friendly biocontrol agent. The incompatible insect technique (IIT) employs the symbiont-associated (e.g., *Wolbachia*) reproductive incompatibility as a biopesticide for the control of insect pests and disease vectors (Fig. 2b). The approach is quite similar to SIT and includes repeated, inundative releases of sterile males in the targeted field population (Berasategui et al. 2016; Bourtzis 2008; Zabalou et al. 2004, 2009). Intensive research in IIT has been performed for several insect pests and disease vectors including *Ceratitis capitata*, *Rhagoletis cerasi*, the tsetse fly, *Culex pipiens*, *Aedes albopictus* and *Culex quinquefasciatus* (Alam et al. 2011; Atyame et al. 2011, 2015; Bourtzis et al. 2014; Neuenschwander et al. 1983; Zabalou et al. 2004, 2009; Zhang et al. 2015a), and

significant attempts to use IIT against wild populations of disease vectors have been applied for the mosquito *Aedes polynesiensis* (O'Connor et al. 2012).

One of the main points of this technique is that, contrary to SIT that allows both sexes to be released as long as they are sterile, this is not possible for IIT which requires strict male release. Indeed, the accidental release of females infected by *Wolbachia* may result in the replacement of the targeted population by a population carrying the *Wolbachia* infection. Providing that IIT produced females are compatible with the wild males, the success of IIT could be compromised, since the *Wolbachia*-infected females would be compatible with either the wild or the released males (Berasategui et al. 2016; Bourtzis 2008; O'Connor et al. 2012). Therefore, IIT requires the development of an efficient method for sexing in order to strictly release infected males. Sexing can be achieved by different techniques like phenotypic sorting or genetic-sexing methods based on classical genetic or molecular methods. However, these separation methods are not available for all target species. In addition, there are concerns about the use of GMOs in the European Union.

Although *Wolbachia* infections are highly prevalent in the drosophilids, CI is not induced by all *Wolbachia* strains (Hoffmann et al. 1996). However, there is evidence that *Wolbachia* can engage in mutualistic relationships (Zug and Hammerstein 2015) and it has been shown to provide a broad spectrum of beneficial effects to its native hosts including protection against viral, microbial, fungal pathogens and parasitoids (Bian et al. 2013; Cattel et al. 2016b; Fytou et al. 2006; Kambris et al. 2010; Martinez et al. 2012, 2014; Moreira et al. 2009; Teixeira et al. 2008; Zindel et al. 2011; Zug and Hammerstein 2015) and increase in host longevity and fecundity (Zug and Hammerstein 2015) which probably explains its pandemic nature (LePage and Bordenstein 2013).

Previous studies reported that *D. sukukii* is infected with a strain of *Wolbachia* called *wSuz* that is present in intermediate prevalence in European and American populations (Cattel et al. 2016a; Hamm et al. 2014; Ometto et al. 2013; Siozios et al. 2013). *wSuz* does not induce a significant level of CI in *D. sukukii* (Cattel et al. 2016a; Hamm et al. 2014). However, *Wolbachia* endosymbionts inducing CI can be introduced into a novel host, either by back-crossing experiments or by transinfection, and express high levels of CI (Zabalou et al. 2008). This concept has been studied in insect pests and disease vectors for the suppression of natural populations (Laven 1967; O'Connor et al. 2012; Zabalou et al. 2004; Zhang et al. 2015b). Research work has been performed in this field for *D. sukukii*, and two *Wolbachia* strains have been identified as potential candidates for developing the IIT in *D. sukukii*. Both strains were identified using the transinfection approach, and they induce a very high level of

CI in this pest regardless of the presence of *wSuz* in females (Mouton et al., personal communication). However, it is critical to address any questions related to host fitness and mating competitiveness of the *Wolbachia*-infected *D. sukuzii* males under semi-field conditions prior to the deployment of this approach to a large-scale operational program (O'Connor et al. 2012; Zhang et al. 2015a). As discussed above for SIT, male competitiveness in an IIT program may be impacted by the rearing methods and processes, cold storage, shipping and release approaches, but also by the introduction of new *Wolbachia* strains as in the case of transinfected lines. A potential IIT application would first and foremost require a thorough biological characterization of the host–bacterial symbiotic association. The *Wolbachia* strain and the host nuclear background are important factors for the expression of CI. Previous studies have suggested that the genetic background of the host is actively involved in the expression of different *Wolbachia* phenotypes, affecting also the *Wolbachia* density. This means that ideally the genomic background of the mass-reared insects should be the same with the one in the target field population. In addition, infection with one or more *Wolbachia* strains could impact the fitness and sexual behavior of host insects, leading in negative effects on the host sexual competitiveness and fitness traits (Bourtzis et al. 2014; Mouton et al. 2007). Given the above, mass-reared insect lines should be evaluated for any potential impacts of the *Wolbachia* symbiont and the genetic variability of the host before including IIT in an integrated control approach.

Combination of SIT/IIT for *D. sukuzii* management

A promising alternative approach for the biological control of *D. sukuzii* is coupling SIT with IIT (Fig. 2c). In general, female insects are more sensitive to radiation than male insects in terms of the induction of sterility, and it may be possible to identify a minimum dose of radiation that leads to complete sterility in females (Bourtzis and Robinson 2006; Zhang et al. 2015a, 2016). As a result, any accidentally released *Wolbachia*-infected females will be sterile and the risk of population replacement is reduced (Bourtzis et al. 2014, 2016; Brelsfoard et al. 2009; Lees et al. 2015). In such a system, the released cytoplasmically incompatible males could also receive a low dose of radiation to ensure complete sterility of females that were not removed. In this case, the sterility of released males would be due to both *Wolbachia* and irradiation, while the female sterility would only be caused by irradiation. Stress accumulated throughout the rearing, handling, storage, transport and release processes may affect the biological quality of the released males (Dyck et al. 2005). Less competitive males in the field would result in lower induction of sterility in the field females. The combination of SIT with IIT may offer a way out of this,

as the released males will be infected with *Wolbachia* and thus a lower irradiation dose can be applied that will allow for more competitive males. This combined strategy could in principle be applied to any targeted species for which an adequate sexing system is not available. Integration of such a protocol combining low irradiation dose with CI has proved to be an efficient strategy in programs targeting the population suppression of *Aedes albopictus* (Zhang et al. 2015a, b, 2016).

Requirements for SIT and/or IIT

Before the application of an SIT and/or IIT program against *D. sukuzii*, it is, nevertheless, important to consider potential limiting factors that may render the program ineffective.

Laboratory domestication and mass rearing

Apart from the factors affecting mass rearing, the process itself requires industrial-scale equipment and protocol that will allow for the mass production of high-quality sterile *D. sukuzii* insects in a cost-effective manner. Another challenge is to develop an economic viable artificial larval and adult diet. The quality of the sterile insects should be continuously monitored to ensure that the desirable biological traits are maintained.

Rearing is a crucial step for SIT and IIT, and the initial fly material used in the rearing process as well as the genomic changes/adaptations is important factors regarding biological quality and consequently release of the manipulated fly specimens (Gilchrist et al. 2012). It is known that within some generations populations can adapt to the mass-rearing environment producing individuals which may significantly differ from their wild counterparts (Gilchrist et al. 2012; Gilligan and Frankham 2003). Several life history traits could be affected during the laboratory adaptation process including reduction in developmental time, lifespan, dispersal ability and stress resistance, as well as early fertility and increased fecundity (Gilchrist et al. 2012; Hoffmann et al. 2001; Raphael et al. 2014). Several studies have reported that genetic diversity loss may occur rapidly during the early generations of domestication (Gilchrist et al. 2012; Raphael et al. 2014; Zygouridis et al. 2014). Results from Gilchrist et al. (2012) in *Bactrocera tryoni* concur with other studies in *Bactrocera oleae* documenting the loss of genetic diversity in captive populations (Zygouridis et al. 2014). This genetic issue and the associated phenotypic effects may severely compromise the success of SIT and IIT programs as the fly quality in the *D. sukuzii* mass-rearing facility may be severely jeopardized. Thus, it is required to develop a strategy that will allow maintaining genetic diversity, biological quality and competitiveness.

Symbiotic bacteria have shown to affect several aspects of the biology, physiology, nutrition and ecology including reproduction and mating behavior of their insect hosts in diverse ways (Augustinos et al. 2015; Bourtzis and Miller 2003, 2006, 2009; Douglas 2011; Eleftherianos et al. 2013; Engel and Moran 2013; Koukou et al. 2006; Miller et al. 2010; Sharon et al. 2010; Weiss and Aksoy 2011; Zchori-Fein and Bourtzis 2011). *Drosophila* species is associated with taxonomically restricted microbial communities compared to mammals, with only four bacterial families being the dominant taxa (Broderick and Lemaitre 2012; Chandler et al. 2011; Corby-Harris et al. 2007; Erkosar et al. 2013; Wong et al. 2013). Several factors influence the microbiota composition including environmental conditions and habitats, life cycle stages, host age and more especially diet (Chandler et al. 2011; Erkosar et al. 2013; Staubach et al. 2013; Yun et al. 2014). Diet proved to be a driving factor in shaping the gut microbiome diversity (Chandler et al. 2011). A specific diet determines which microbes are able to colonize this environment. As a result, most of the bacteria characterized in laboratory-reared *Drosophila* populations are not the most abundant in wild populations, and vice versa (Chandler et al. 2011; Staubach et al. 2013). These observations may explain why the fitness of some laboratory-adapted populations is not comparable to that of natural populations.

The biological quality of the mass-produced insects is of major importance for SIT and IIT applications, and its improvement in a mass-rearing facility would result in enhancement of the efficacy of SIT and/or IIT applications. Mass rearing and/or irradiation may affect the gut bacterial community structure of insects, and this may also impact mating competitiveness of sterile males (Ben Ami et al. 2010; Hamden et al. 2013). Similarly, several studies have shown that *Wolbachia* may be associated with mating isolation phenomena (Koukou et al. 2006; Miller et al. 2010). Since insect-associated microbiota seems to play a major role in fly quality, it is important to identify the factors that could alter and/or modify the composition of the intestinal symbionts and consequently reduce the overall fitness of the sterile males. In addition, the use of probiotics (originating from endogenous gut-associated bacterial species) should be explored, as a means to improve rearing efficiency and mating competitiveness as shown for medfly (Augustinos et al. 2015; Gavriel et al. 2011).

Irradiation protocol

Irradiation dose required for complete male sterility may affect biological quality and mating competitiveness of *D. suzukii*. The optimal conditions, developmental stage and dose for irradiation-induced male sterility should be determined to minimize potential negative effects, while the use

of probiotics could ameliorate them as shown for medfly (Gavriel et al. 2011). Applying the SIT to *D. suzukii* involves irradiation during a narrow time window at the late pupal stage to induce atrophy of the reproductive organs, therefore inducing reproductive sterility without affecting reproductive behavior, and then release into the target area where the sterile males sexually compete with their wild counterparts. An irradiation protocol must be thoroughly developed and tested to ensure a high degree of confidence that the process will properly sterilize the insects. For the combined SIT/IIT approach, it is also important to determine the minimum optimal dose for complete sterilization of female *D. suzukii* that will not influence the male mating competitiveness. Therefore, it is important that this dose is significantly below that normally required for full male sterility (Lees et al. 2015; Zhang et al. 2016).

The SIT and IIT programs also need to ensure that, once in the field, the sterile males compete effectively with wild males and mate with wild females and successfully transfer their sperm. Effective methods for monitoring and providing timely feedback on the quality and competitiveness of sterile fruit flies are critical to the success of SIT programs. The quality of the sterile mass-reared insects and the mating competitiveness—as measured by their ability to induce sterility in field females—with the wild counterparts are critical factors that should be measured and assessed using appropriate procedures (Dyck et al. 2005). Quality control protocols at laboratory and semi-field conditions level are required for the evaluation of flight ability and effectiveness of the mass-reared, irradiated and released sterile *D. suzukii*. The Joint FAO/IAEA Insect Pest Control Laboratory has developed a quality control manual for fruit flies (FAO/IAEA/USDA 2014). The manual includes procedures for product quality control (QC) for mass-reared and sterilized flies as well as handling and packaging methods of pupae intended to be used in SIT programs. These procedures involve series of tests that measure pupae weight, emergence, sterility, longevity, flight ability, sexual competitiveness and survival under stress. The quality of the mass-produced sterile insects will determine the ratio required for the population suppression.

Management of thermal tolerance

Low-temperature treatment is an integral part of the rearing or release protocols in IPM programs (Colinet and Boivin 2011; Enkerlin 2007). For both SIT and IIT, efficient deployment of insects is achieved when their release coincides with the appearance of the pests, and there is often a timing gap between production and release. The ability to cold-store insects without loss of performance and to mobilize them quickly upon demand is thus essential for a viable biological control using SIT and/or IIT. Mass-reared insects are often

exposed to low temperature for immobilization and handling during rearing process. Long-distance shipping from rearing facilities to release sites is also performed under low temperature. Finally, temperature within the release site (e.g., greenhouse) may contrast with rearing conditions and may be stressful to released insects (being too high or too low). This latter issue can be mitigated by application of thermal conditioning protocols before release to prevent thermal stress-induced mortality. In consequence, a successful application of SIT and/or IIT requires large basic knowledge on thermal biology of the target insect in order to develop protocols to manipulate its thermal tolerance.

Most recent studies on *D. suzukii* cold tolerance were designed to understand overwintering strategy in newly infested cold regions, in order to better predict invasion potential or winter survival probability (Dalton et al. 2011; Rossi-Stacconi et al. 2016; Shearer et al. 2016; Stephens et al. 2015; Wallingford and Loeb 2016; Zerulla et al. 2015). The recent literature shows that *D. suzukii* is freeze intolerant and chill susceptible (Dalton et al. 2011; Jakobs et al. 2015; Kimura 2004; Plantamp et al. 2016; Ryan et al. 2016), but possesses a large thermal tolerance plasticity, which likely favors its overwintering success (Jakobs et al. 2015). This large plasticity could be exploited to modulate *D. suzukii* thermal tolerance via classical acclimation protocols, e.g., pre-exposure to sublethal conditions (Colinet and Hoffmann 2012). *Drosophila suzukii* is supposed to overwinter as adult dark winter morph (Kanzawa 1936; Shearer et al. 2016; Stephens et al. 2015; Toxopeus et al. 2016; Wallingford et al. 2016). This morph is characterized by an arrest of reproduction and an increased cold tolerance (Shearer et al. 2016; Stephens et al. 2015; Toxopeus et al. 2016; Wallingford and Loeb 2016), but it is not yet clear whether this morph entails a true reproductive diapause or not (Rossi-Stacconi et al. 2016; Toxopeus et al. 2016; Wallingford et al. 2016; Zhai et al. 2016). Understanding how to initiate and arrest its diapause may provide valuable tools for long-term storage. Knowledge of the overwintering biology of *D. suzukii* is also a crucial factor in predicting the size of the summer population in a given area (Rossi-Stacconi et al. 2016). This will allow for more efficient planning of the control methods implementation. For instance, the SIT and IIT approaches that are based on the release of mass-produced sterile males could take advantage of the shortage of wild males during late winter/early spring periods. Their implementation at those periods would reduce the competition from the wild males and increase the mating frequency for the sterile males (Rossi-Stacconi et al. 2016). Given the high level of inconsistency in the available data regarding *D. suzukii* mortality at different temperatures, detailed thermo-biological data are highly needed (Asplen et al. 2015). To fully appreciate the innate capacity of *D. suzukii* to cope with both cold and heat stress, a comprehensive approach

based on tolerance landscape has been proposed by Enriquez and Colinet (2017). The authors found that temperatures below 5–7 °C were likely not compatible with cold storage, while temperatures above 32 °C would drastically reduce survival. Thermal survival patterns were also influenced by sex, stage, as well as relative humidity. This basic information is important to develop protocols to manipulate *D. suzukii* thermal tolerance. Undoubtedly, studying thermal biology of *D. suzukii* is essential to facilitate the application of SIT and/or IIT, but other aspects of SIT and/or IIT could be impacted (positively or negatively) by temperature. For instance, lowering metabolic rate with low temperature or promoting generic mechanisms of stress tolerance with pre-exposures may mitigate off-target irradiation damages and promote post-irradiation performance. Applying mild thermal stress (heat or cold) at some specific stage could trigger antioxidant defenses and lower oxidative damages resulting from irradiation. Such a hormesis approach (i.e., physiological conditioning) has been observed when applying short-term anoxic conditions before irradiation in the Caribbean fruit fly (López-Martínez and Hahn 2014). Besides, thermal conditions may directly affect *Wolbachia* load (Mouton et al. 2006, 2007), and consequently, it is important to analyze whether thermal treatments allow maintenance of high level of CI. On the other hand, data coming from the study of Neelakanta et al. (2010) have shown that gut symbionts can enhance the ability of insects to tolerate cold temperatures and overwinter. Acquiring knowledge on basal and induced thermal tolerance of irradiated and *Wolbachia*-infected *D. suzukii* will also help in defining accurate management practices based on SIT and/or IIT programs.

Conclusions

Drosophila suzukii has spread across Europe and causes significant economic losses in commercial soft fruits. Its rapid invasive rate in the continent poses a challenge to the development of efficient monitoring and management tools. *Drosophila suzukii* is a highly prolific species with exceptional biological traits that contribute to its persistence in distinct geographic areas. This fact, combined with its extreme polyphagy behavior, suggests that an AW-IPM program is required for the effective control of the pest. Chemical control tactics are currently the most widespread method used to control *D. suzukii*. Insect resistance to chemicals, frequent applications of insecticides owing to *D. suzukii*'s short generation time and concerns about public health are considerable issues that have turned research toward non-chemical, environmentally sound and sustainable approaches. Investment in innovative biological control methods could lead to a reduction in *D. suzukii*'s populations not only in cultivated crops, but also in natural niches that are normally neglected

by chemical approaches. Development of SIT for *D. suzukii*, as a component of an AW-IPM approach, is expected to significantly contribute to population management, especially in greenhouses and other confined areas, such as exclusion netting high tunnels. Driven by the encouraging results of SIT applications on other pests, research groups have focused their efforts on addressing the challenges related to the method. In the unlikely event that the irradiated sterile males present decreased or insufficient mating competitiveness, employment of IIT as a supplement to SIT is worth considering. This combined strategy could offer a way out of dead ends posed by each method individually. Management of thermal tolerance is a crucial factor that allows cold storage of insects without loss of their performance. Acquiring knowledge on thermal biology of *D. suzukii* will allow the deployment of thermal tolerance manipulation protocols. As soon as these protocols are mature, the rearing and release processes of SIT or the combined SIT/IIT approaches will profit from them and become more reliable. Whatever the method to be applied, there are several questions that need to be addressed beforehand and knowledge gaps to shed light on. To this aim, research should be carried out to deploy accurate and well-established management programs to decrease the impact of this invasive pest.

Author contributions

KN and KB organized the review. KN wrote the review, and HC contributed in the “Management of thermal tolerance” part. All authors reviewed and provided constructive comments for this manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with animals performed by any of the authors.

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Annexe 2

Figures et tableaux supplémentaires de l'article 1 :

Basal tolerance to heat and cold exposure of the spotted wing drosophila, *Drosophila suzukii*

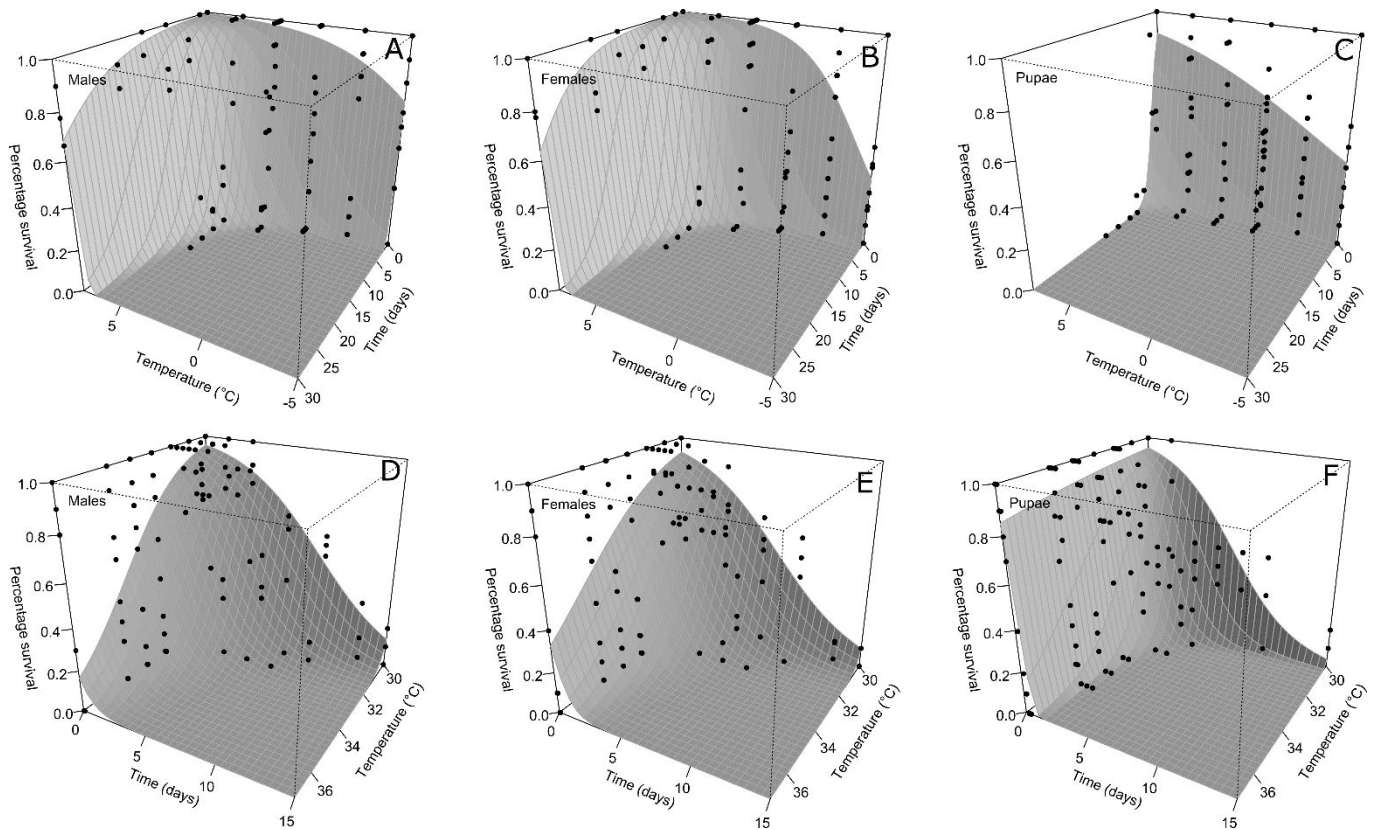


Figure S1 : Thermal tolerance landscapes for low and high temperatures.

Males (A), females (B) and pupal (C) cold tolerance landscapes, and Males (D), females (E) and pupal (F) heat tolerance landscapes. Points are observed values, and surfaces correspond to GLMs predictions (Binomial GLM, link = logit).

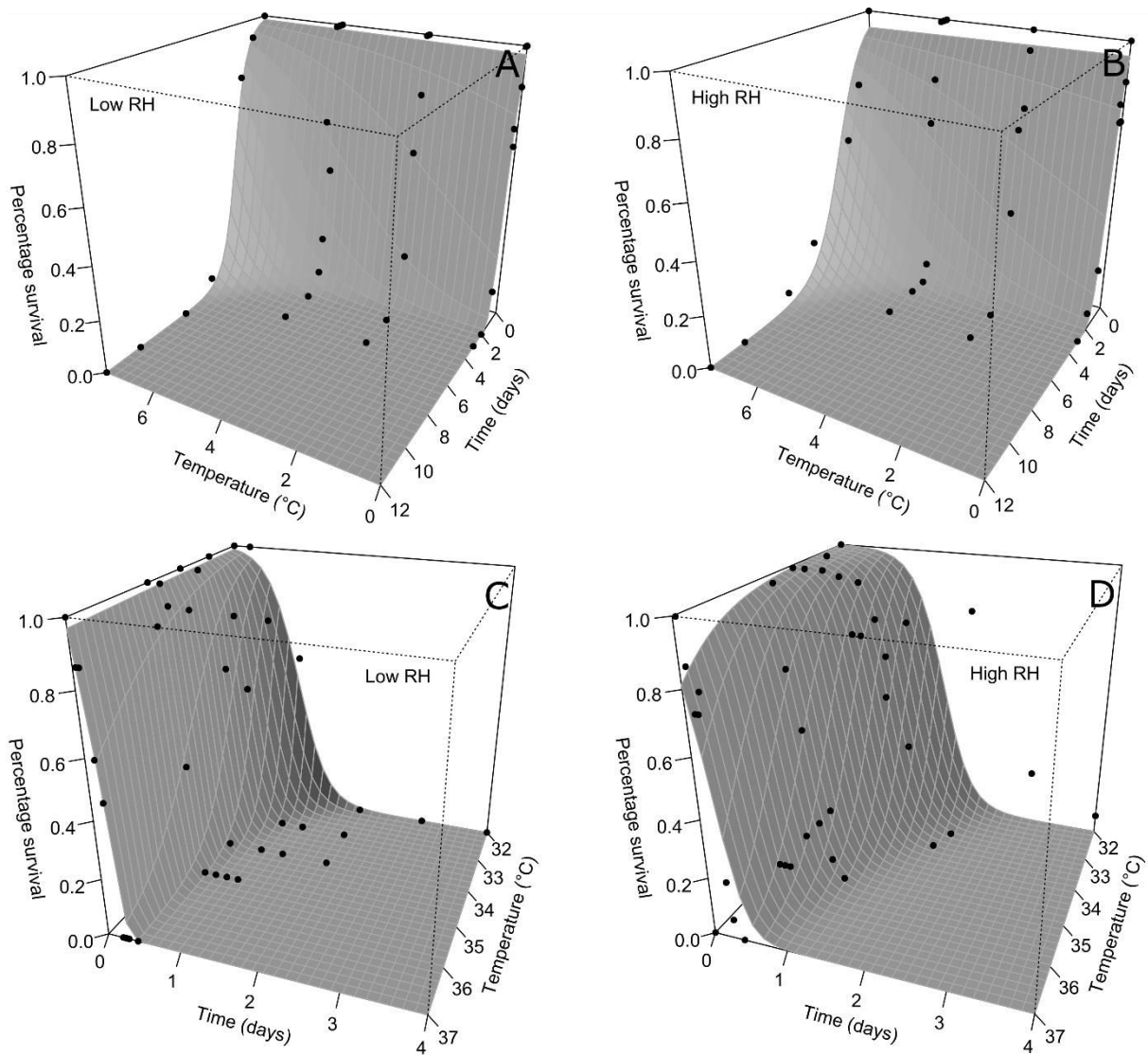


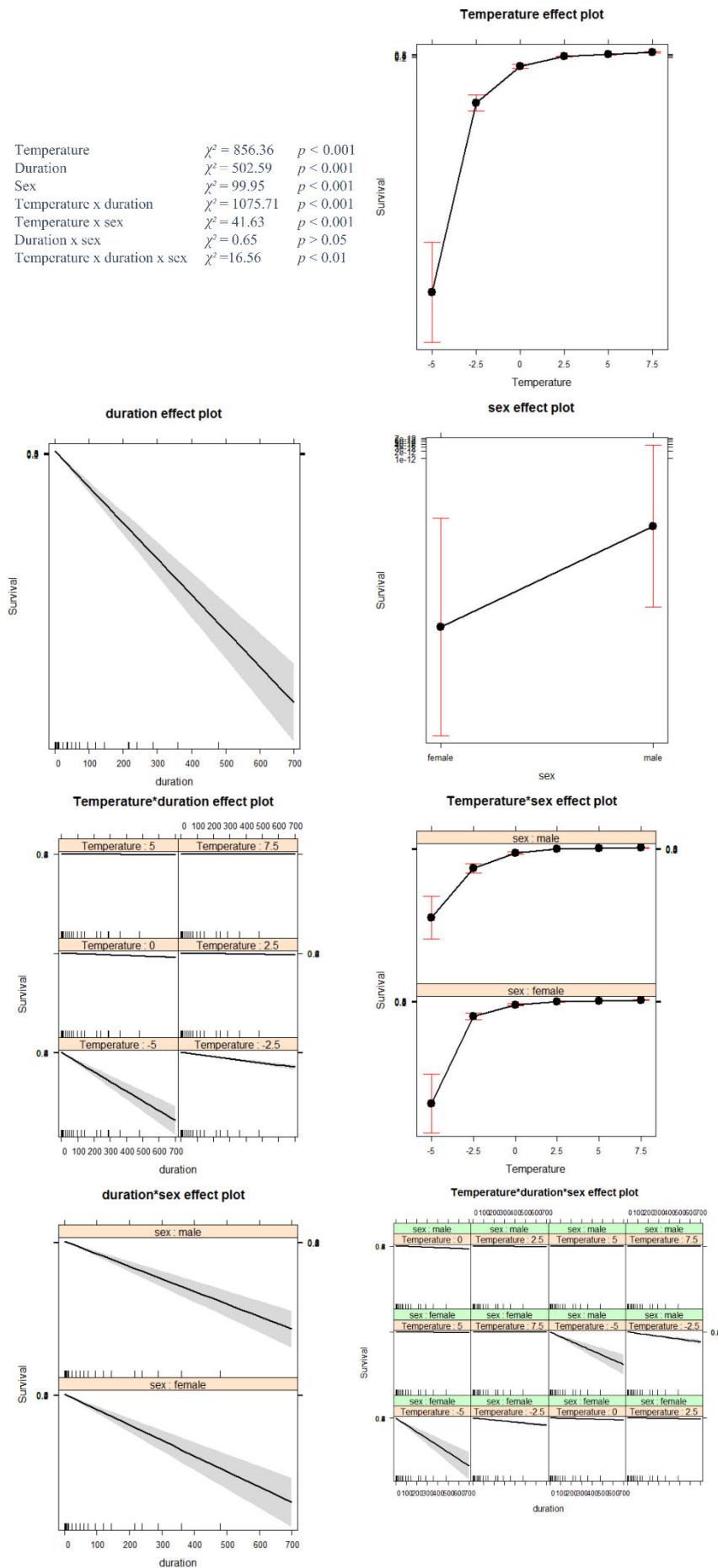
Figure S2 : Thermal tolerance landscapes for low and high temperatures under two relative humidity (RH) levels.

Cold tolerance landscapes under low (A) and high (B) relative humidity, and heat tolerance landscapes under low (C) and high (D) relative humidity. Points corresponds to observed values, and surfaces corresponds to GLMs predictions (Binomial GLM, link = logit).

Temperature	$\chi^2 = 856.36$	$p < 0.001$
Duration	$\chi^2 = 502.59$	$p < 0.001$
Sex	$\chi^2 = 99.95$	$p < 0.001$
Temperature x duration	$\chi^2 = 1075.71$	$p < 0.001$
Temperature x sex	$\chi^2 = 41.63$	$p < 0.001$
Duration x sex	$\chi^2 = 0.65$	$p > 0.05$
Temperature x duration x sex	$\chi^2 = 16.56$	$p < 0.01$

Figure S3: Effect plots from GLM on the experiment with adults exposed to low temperatures.

The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are cold exposure temperature, duration, sex (Male vs Female) and all the interactions. The statistical outputs (from the table of deviance) are also given (in blue) before the plots for all terms of the model.



Temperature $\chi^2 = 156.68$ $p < 0.001$
 Duration $\chi^2 = 463.94$ $p < 0.001$
 Temperature x duration $\chi^2 = 161.43$ $p < 0.001$

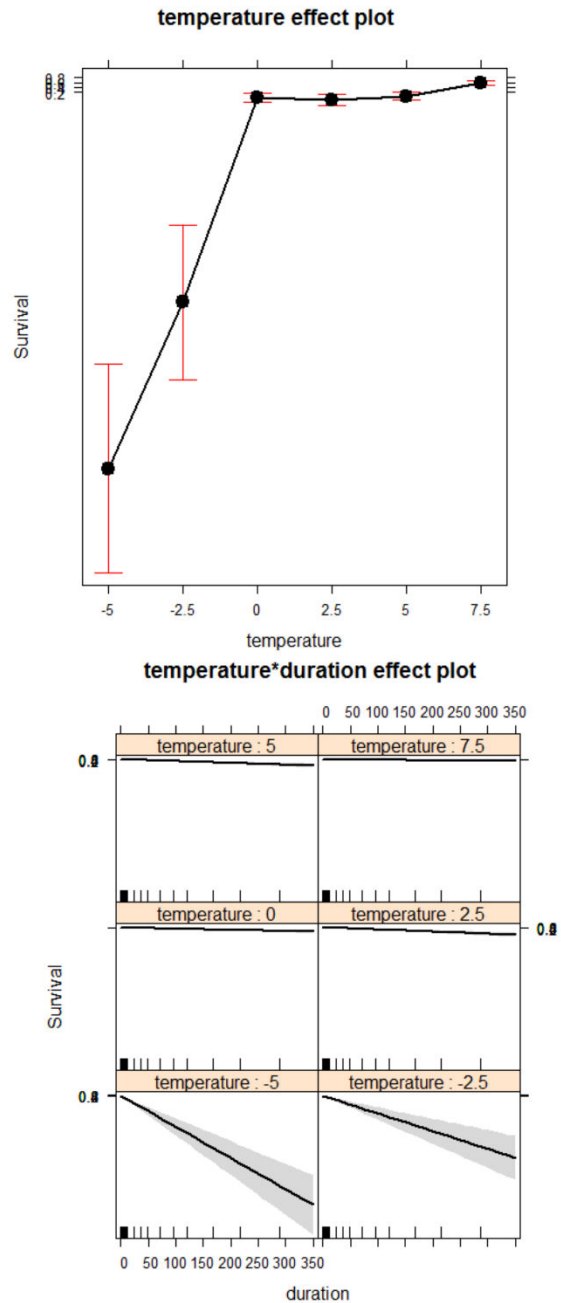


Figure S4

Effect plots from GLM on the experiment with pupae exposed to low temperatures.

The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are cold exposure temperature, duration, and all the interactions. The statistical outputs (from the table of deviance) are also given (in blue) before the plots for all terms of the model.

Temperature	$\chi^2 = 819.69$	$p < 0.001$
Duration	$\chi^2 = 889.77$	$p < 0.001$
Sex	$\chi^2 = 1.52$	$p > 0.05$
Temperature x duration	$\chi^2 = 1495.20$	$p < 0.001$
Temperature x sex	$\chi^2 = 94.43$	$p < 0.001$
Duration x sex	$\chi^2 = 0.19$	$p > 0.05$
Temperature x duration x sex	$\chi^2 = 10.99$	$p > 0.05$

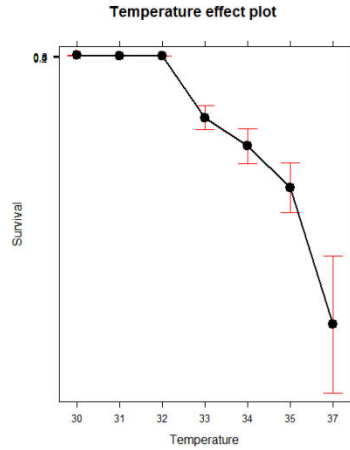
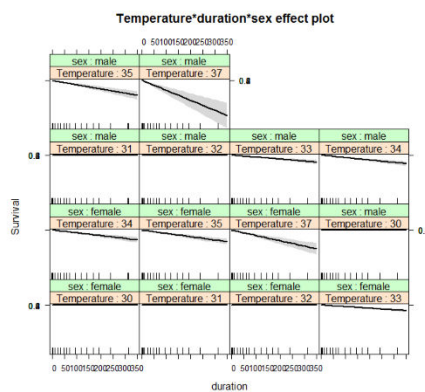
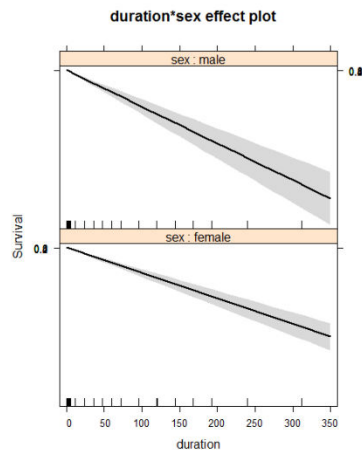
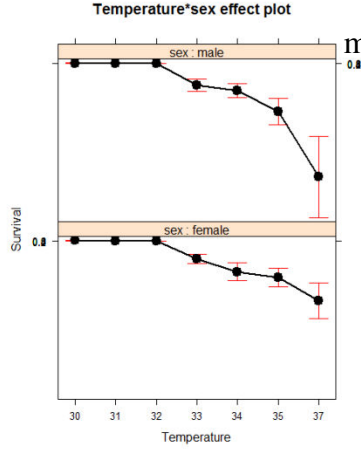
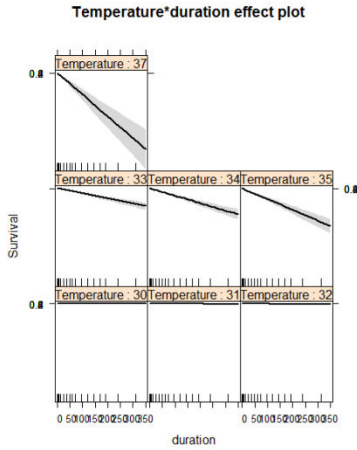
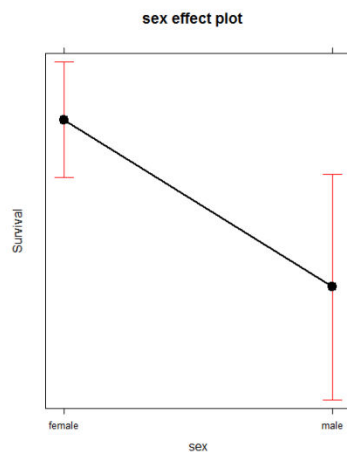
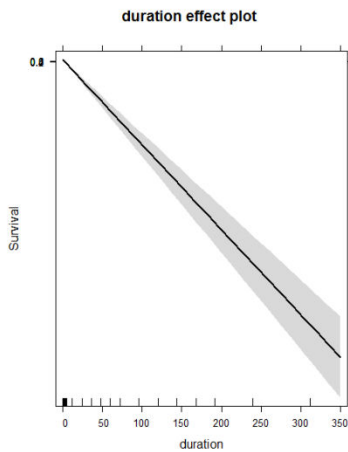


Figure S5: Effect plots from GLM on the experiment with adults exposed to high temperatures.

The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are heat exposure temperature, duration, sex (Male vs Female) and all the interactions. The statistical outputs (from the table of deviance) are also given (in blue) before the plots for all terms of the model.



Temperature	$\chi^2 = 210.72$	$p < 0.001$
Duration	$\chi^2 = 388.71$	$p < 0.001$
Temperature x duration	$\chi^2 = 662.25$	$p < 0.001$

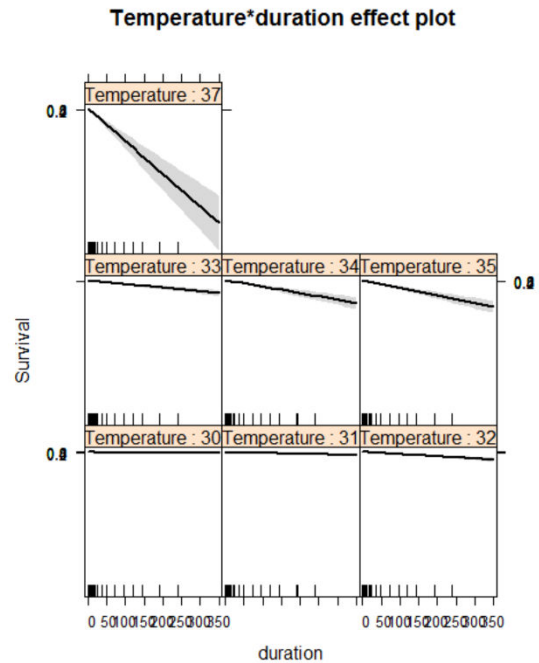
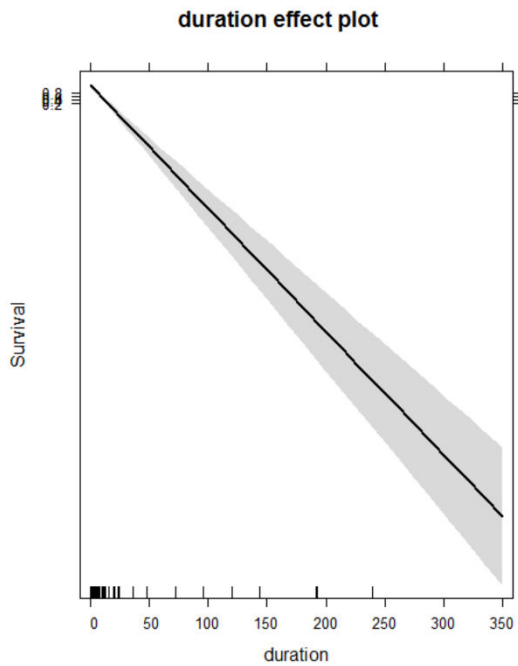
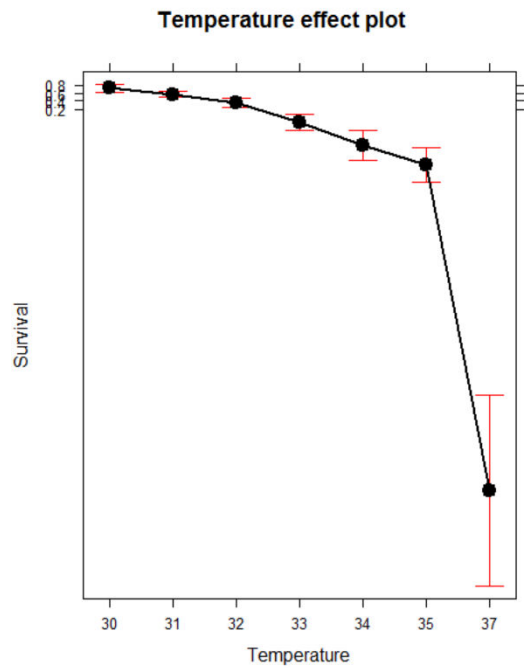


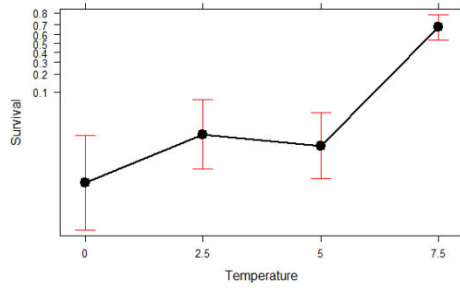
Figure S6

Effect plots from GLM on the experiment with pupae exposed to high temperatures.

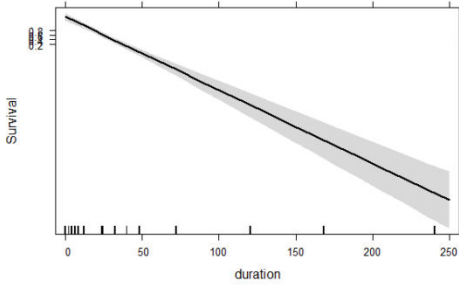
The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are heat exposure temperature, duration and all the interactions. The statistical outputs (from the table of deviance) are also given (in blue) before the plots for all terms of the model.

Temperature	$\chi^2 = 91.74$	$p < 0.001$
Duration	$\chi^2 = 694.88$	$p < 0.001$
RH	$\chi^2 = 0.02$	$p > 0.05$
Temperature x duration	$\chi^2 = 68.07$	$p < 0.001$
Temperature x RH	$\chi^2 = 5.05$	$p > 0.05$
Duration x RH	$\chi^2 = 5.48$	$p < 0.05$
Temperature x duration x RH	$\chi^2 = 7.67$	$p > 0.05$

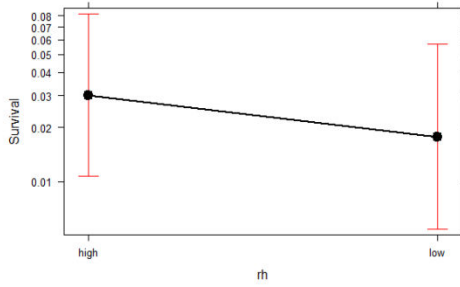
Temperature effect plot



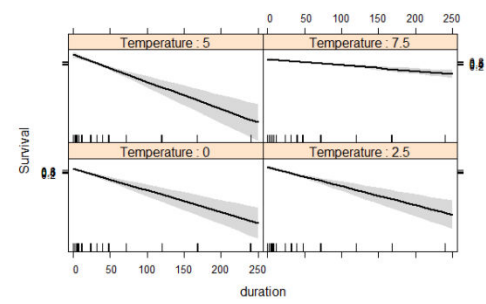
duration effect plot



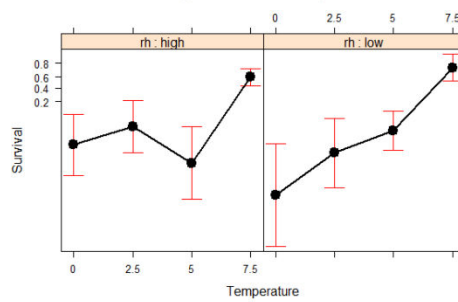
rh effect plot



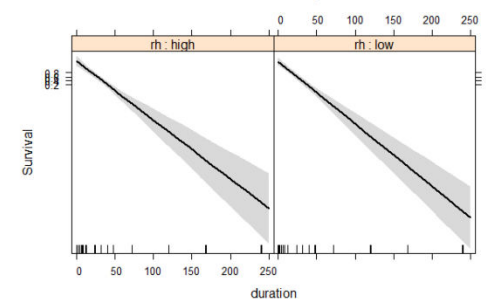
Temperature*duration effect plot



Temperature*rh effect plot



duration*rh effect plot



Temperature*duration*rh effect plot

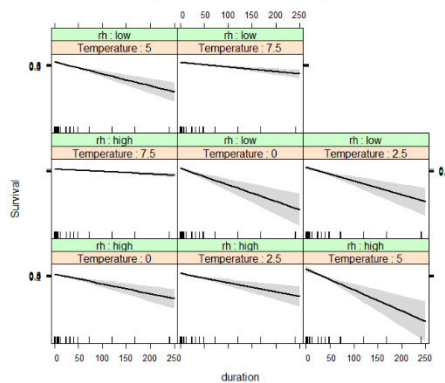


Figure S7: Effect plots from GLM on the experiment with pupae exposed to low temperatures under two relative humidity levels.

The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are cold exposure temperature, duration, RH and all the interactions. The statistical outputs (from the table of deviance) are also given (in blue) before the plots for all terms of the model.

Temperature	$\chi^2 = 306.20$	$p < 0.001$
Duration	$\chi^2 = 831.90$	$p < 0.001$
RH	$\chi^2 = 95.97$	$p < 0.001$
Temperature x duration	$\chi^2 = 83.46$	$p < 0.001$
Temperature x RH	$\chi^2 = 19.25$	$p < 0.001$
Duration x RH	$\chi^2 = 14.17$	$p < 0.001$
Temperature x duration x RH	$\chi^2 = 19.30$	$p < 0.001$

TEMPERATURE effect plot

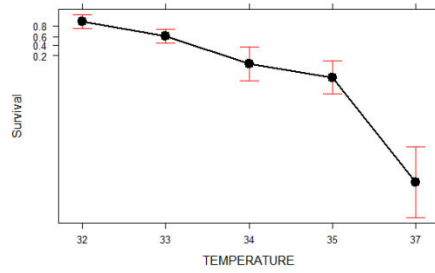
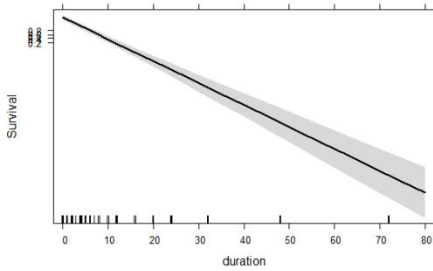
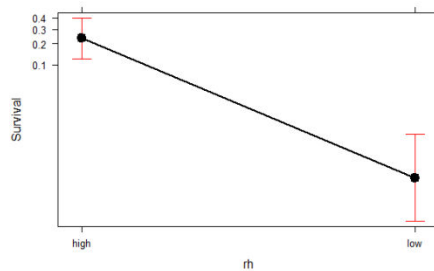


Figure S8: Effect plots from GLM on the experiment with pupae exposed to high temperatures under two relative humidity levels.

duration effect plot

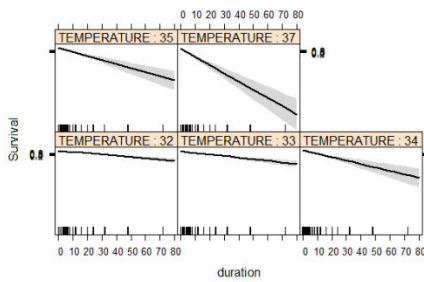


rh effect plot

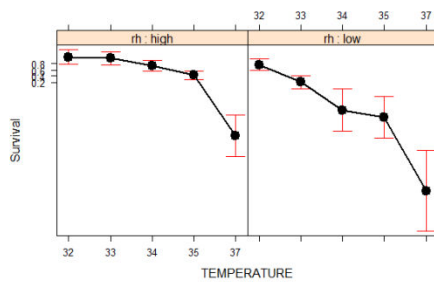


The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are heat exposure temperature, duration, RH and all the interactions. The statistical outputs (from the table of deviance) are also given (in blue) before the plots for all terms of the model.

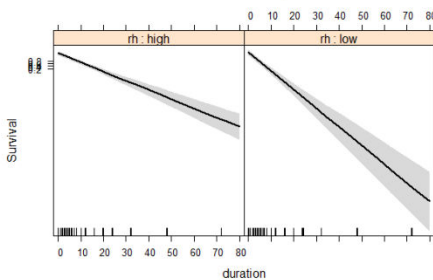
duration*TEMPERATURE effect plot



TEMPERATURE*rh effect plot



duration*rh effect plot



duration*TEMPERATURE*rh effect plot

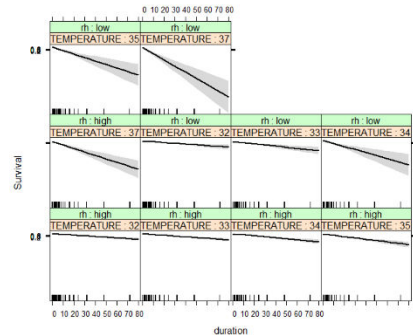


Table S1: Temperatures and respective exposure durations used for adult's cold tolerance assays.

-5 °C	-2.5 °C	0 °C	2.5 °C	5 °C	7.5 °C
20 min	20 min	12 h	12 h	2 d	2 d
40 min	40 min	24 h	24 h	3 d	5 d
60 min	1 h	36 h	36 h	6 d	10 d
80 min	2 h30	48 h	48 h	9 d	15 d
100 min	4 h	60 h	3 d	12 d	20 d
2 h	7 h	3 d	4 d	15 d	30 d
	10 h				
	12 h				

Table S2: Temperatures and respective exposure durations used for pupae's cold tolerance assays.

-5 °C	-2.5 °C	0 °C	2.5 °C	5 °C	7.5 °C
15 min	15 min	2 h	6 h	6 h	1 d
30 min	30 min	4 h	12 h	12 h	2 d
45 min	45 min	8 h	24 h	24 h	5 d
1 h	1 h	12 h	36 h	36 h	7 d
1 h30	1 h30	24 h	3 d	3 d	10 d
2 h	2 h	48 h	4 d	5 d	12 d
2 h30	4 h	72 h			

Table S3: Temperatures and respective exposure durations used for adult's heat tolerance assays.

30 °C	31 °C	32 °C	33 °C	34 °C	35 °C	37 °C
2 d	1 d	12 h	30 min	30 min	20 min	20 min
4 d	2 d	24 h	1 h	1 h	40 min	40 min
7 d	3 d	36 h	2 h	1 h30	60 min	60 min
10 d	4 d	48 h	3 h	2 h	80 min	80 min
13 d	5 d	60 h	4 h	2 h30	100 min	100 min
15 d	6 d	3 d	5 h	3 h	2 h	2 h
	8 d	4 d				
	10 d	6 d				
		8 d				

Table S4: Temperatures and respective exposure durations used for pupae's heat tolerance assays.

30 °C	31 °C	32 °C	33 °C	34 °C	35 °C	37 °C
2 d	12 h	6 h	4 h	2 h	2 h	1 h
4 d	24 h	12 h	8 h	6 h	6 h	2 h
6 d	2 d	24 h	12 h	12 h	12 h	3 h
8 d	3 d	48 h	24 h	24 h	24 h	4 h
10 d	4 d	60 h	36 h	36 h	36 h	5 h
15 d	5 d	4 d	48 h	48 h	48 h	6 h
						7 h
						10 h

Table S5: Temperatures and respective exposure durations used for pupae’s thermal stress assays under high or low relative humidity.

0 °C	2.5 °C	5 °C	7.5 °C	32 °C	33 °C	34 °C	35 °C	37 °C
2 h	6 h	6 h	1 d	6 h	4 h	2 h	2 h	1 h
4 h	12 h	12 h	2 d	12 h	8 h	6 h	6 h	2 h
8 h	24 h	24 h	5 d	24 h	12 h	12 h	12 h	3 h
12 h	36 h	48 h	7 d	48 h	24 h	24 h	16 h	4 h
24 h	3 d	3 d	10 d	3 d	36 h	36 h	20 h	5 h
48 h	4 d	5 d	12 d	4 d	48 h	48 h	24 h	6 h
72 h								7 h
								10 h

Annexe 3

Figures et tableaux supplémentaires de l’article 2 : Effects of fluctuating thermal regimes on cold survival and life history traits of the spotted wing *Drosophila (Drosophila suzukii)*

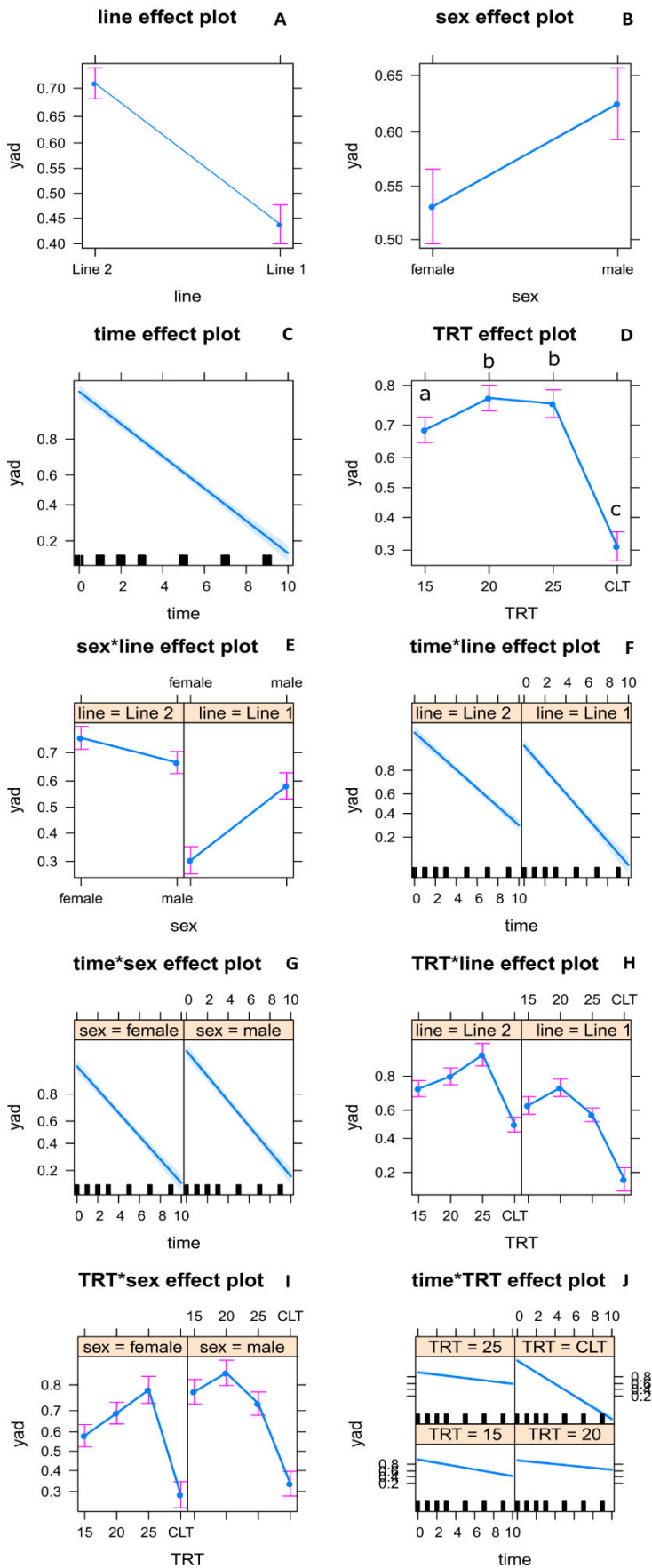


Figure S1: Effect plots from GLM: temperature variation of FTR on adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line and their interactions. Different letters on Fig. S1D correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

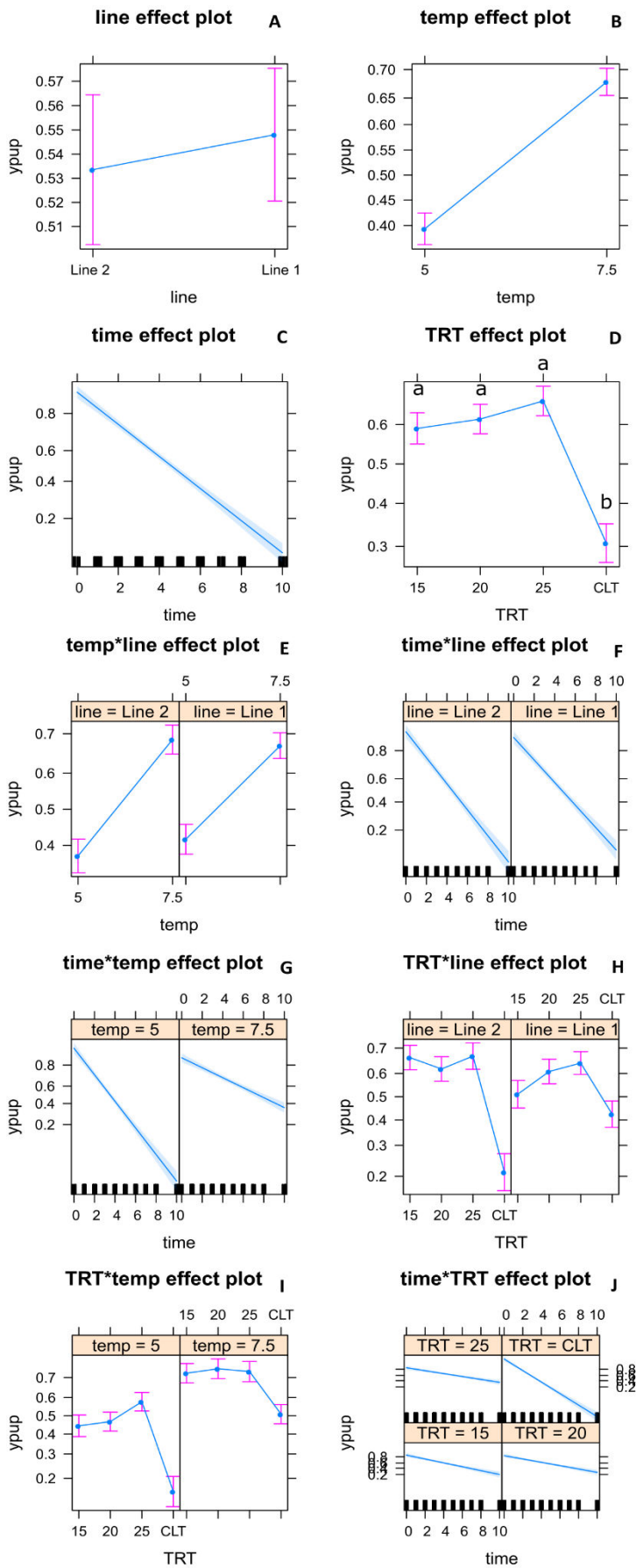


Figure S2: Effect plots from GLM: temperature variation of FTR on pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line and their interactions. Different letters on Fig. S2D correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

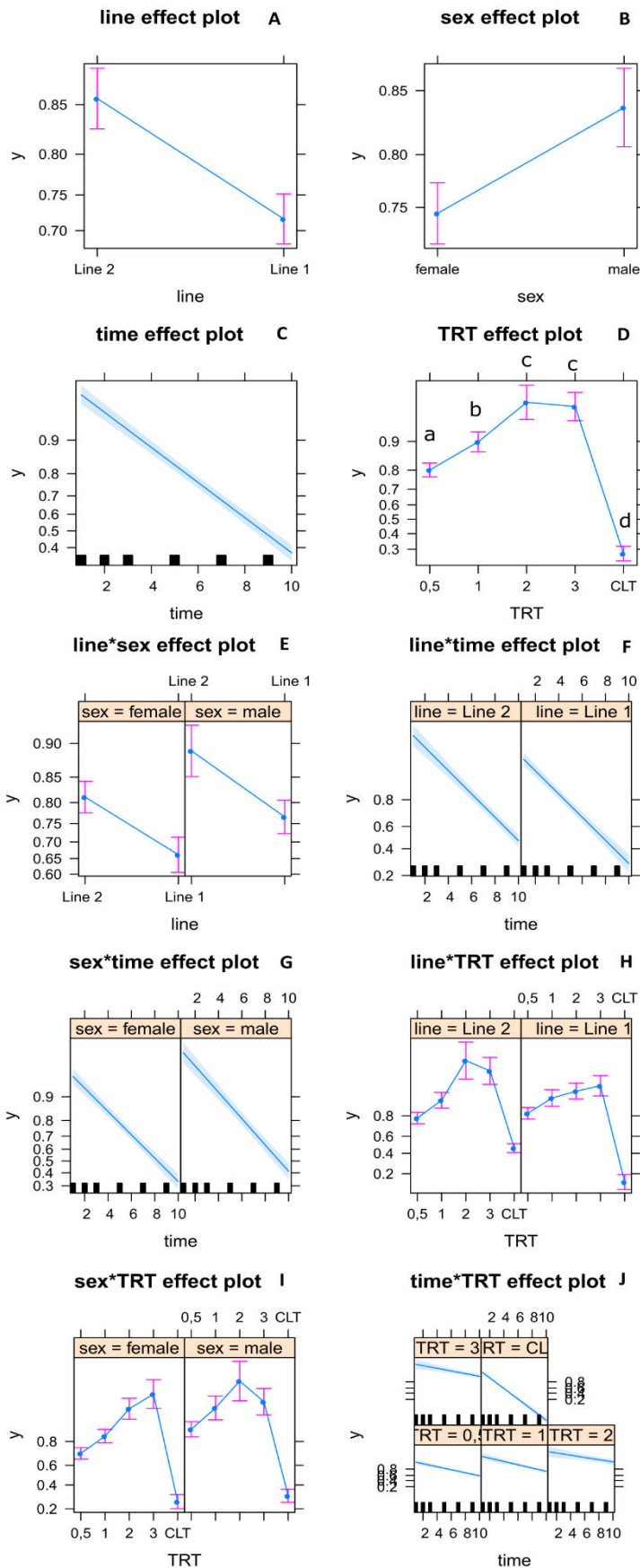


Figure S3: Effect plots from GLM: duration variation of FTR on adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line and their interactions. Different letters on Fig. S3D correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

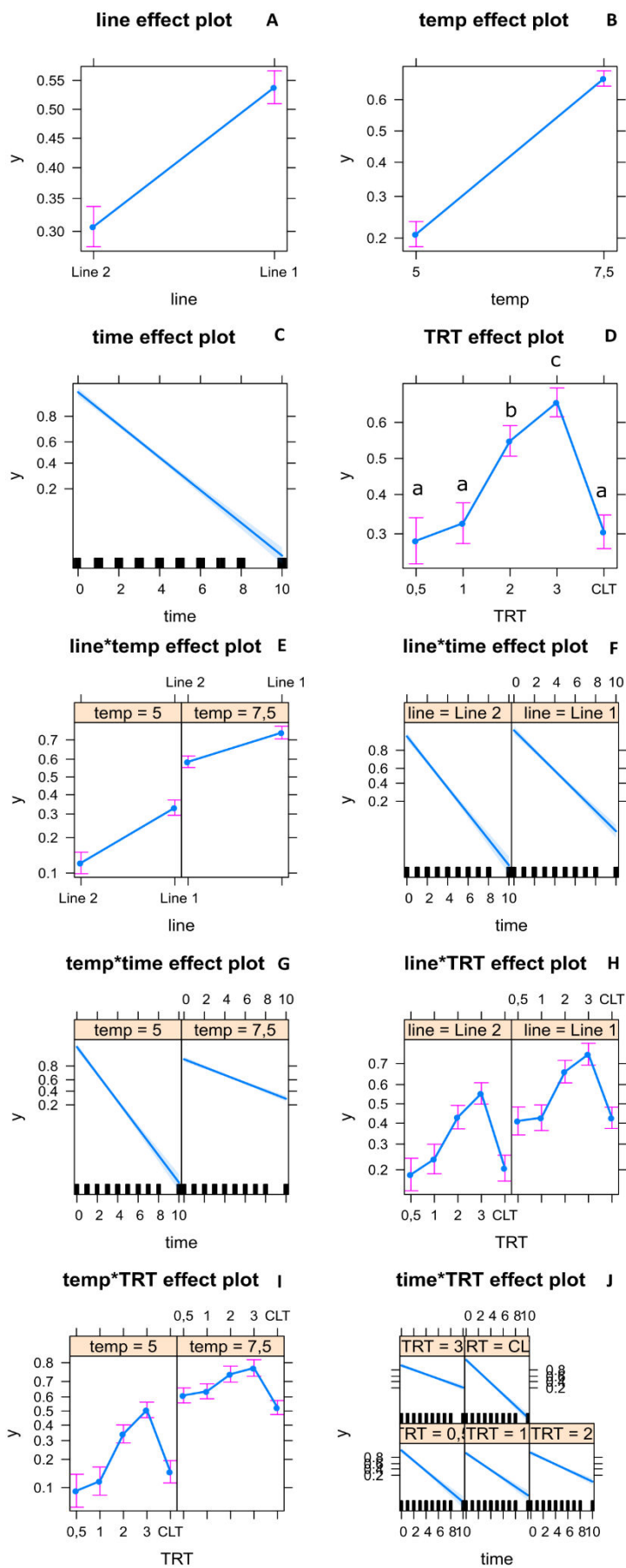


Figure S4: Effect plots from GLM: duration variation of FTR on pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line and their interactions. Different letters on Fig. S4D correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

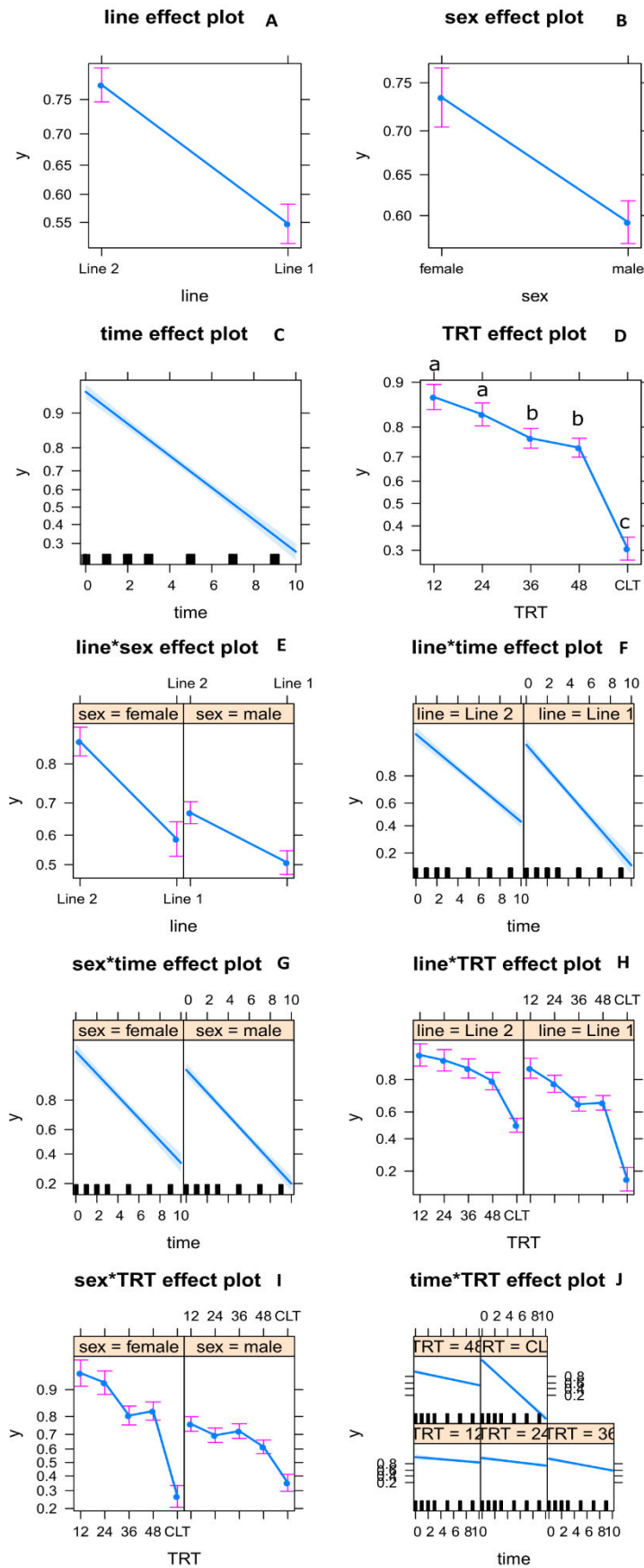


Figure S5: Effect plots from GLM: frequency variation of FTR on adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line and their interactions. Different letters on Fig. S5D correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

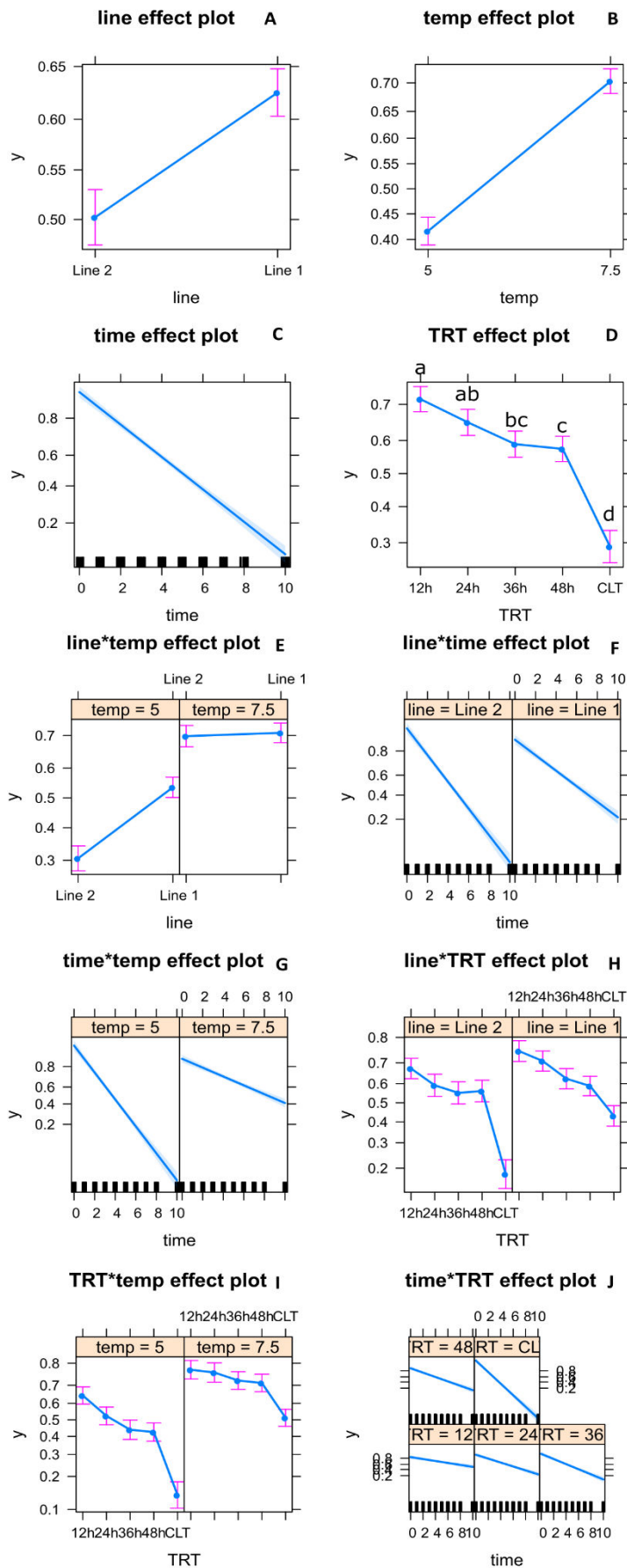


Figure S6: Effect plots from GLM: duration variation of FTR on pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line and their interactions. Different letters on Fig. S6D correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

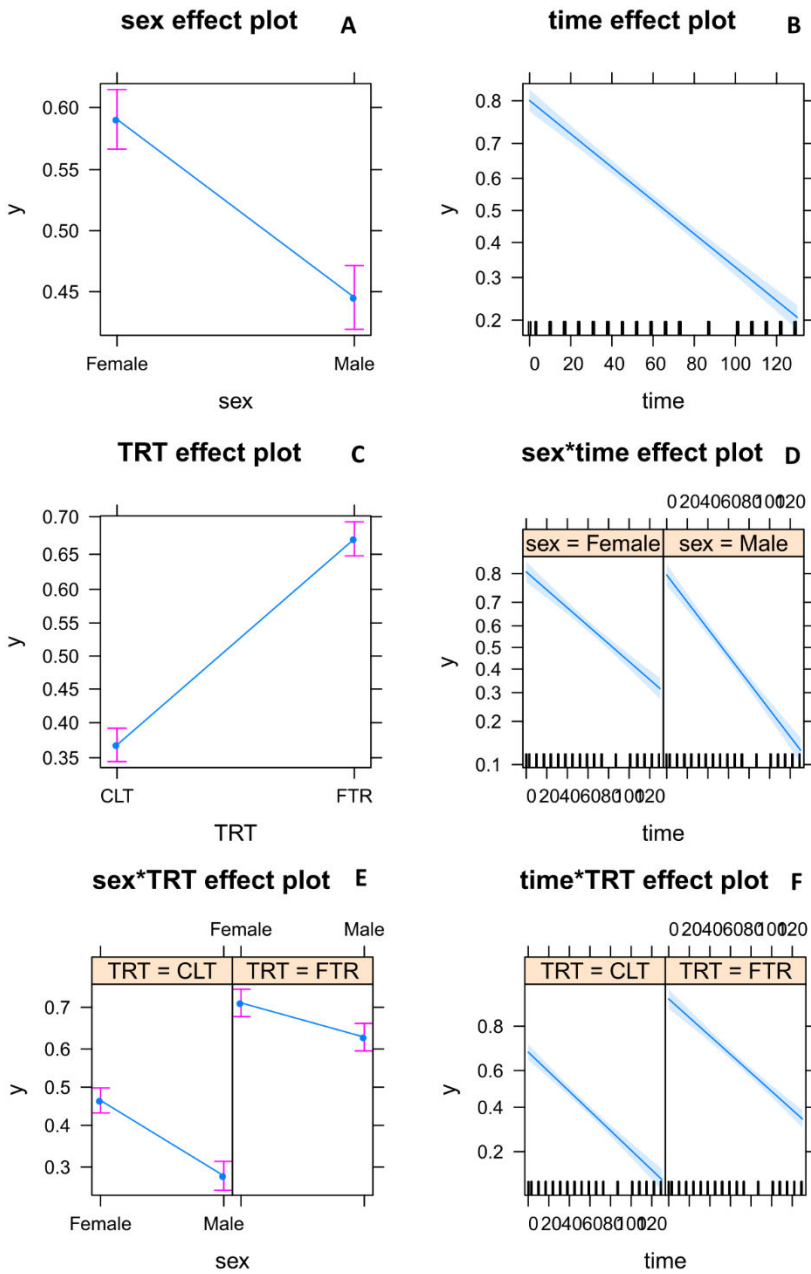
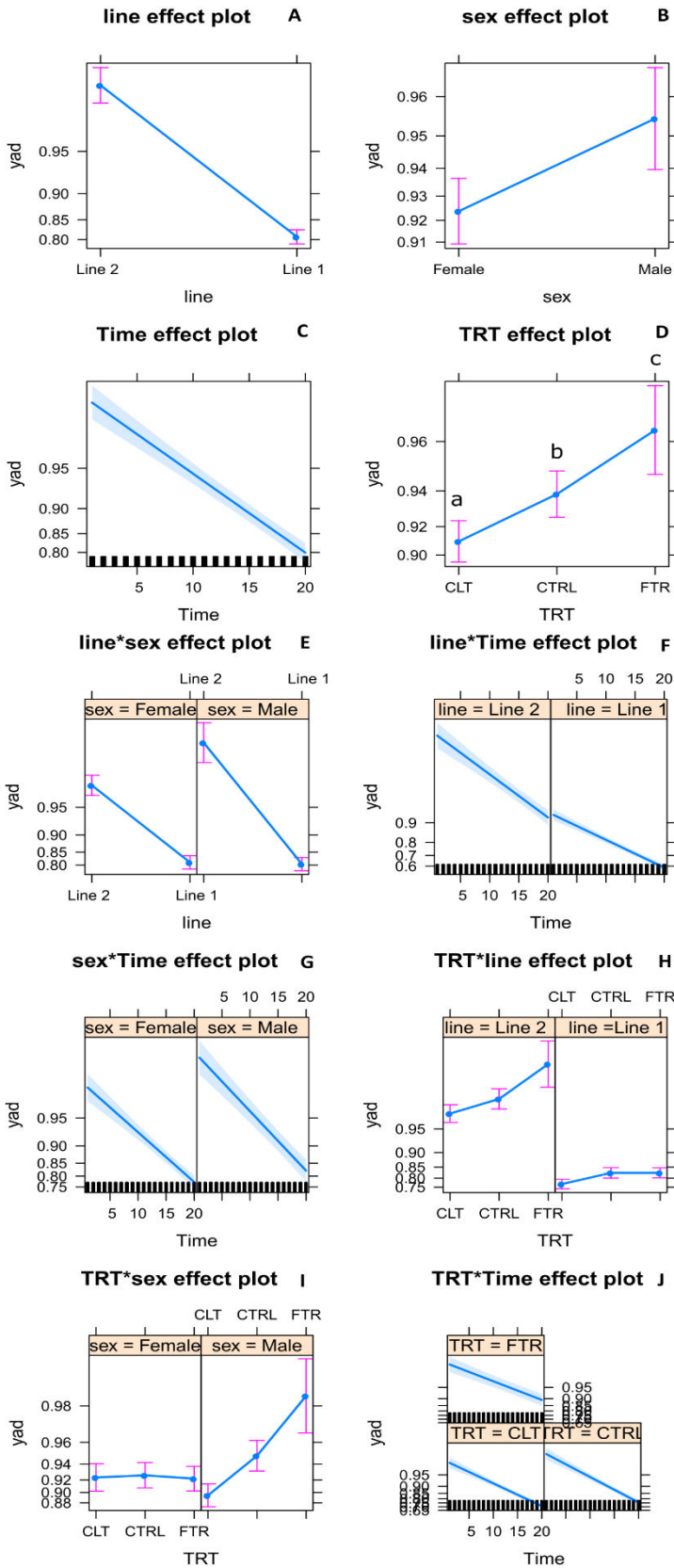


Figure S7: Effect plots from GLM: medium-term cold storage as adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex and their interactions.

Figure S8: Effect plots from GLMM:

Post storage survival of adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line and their interactions. Different letters on Fig. S8D correspond to statistical differences between treatments (least-squares means, $p < 0.05$).



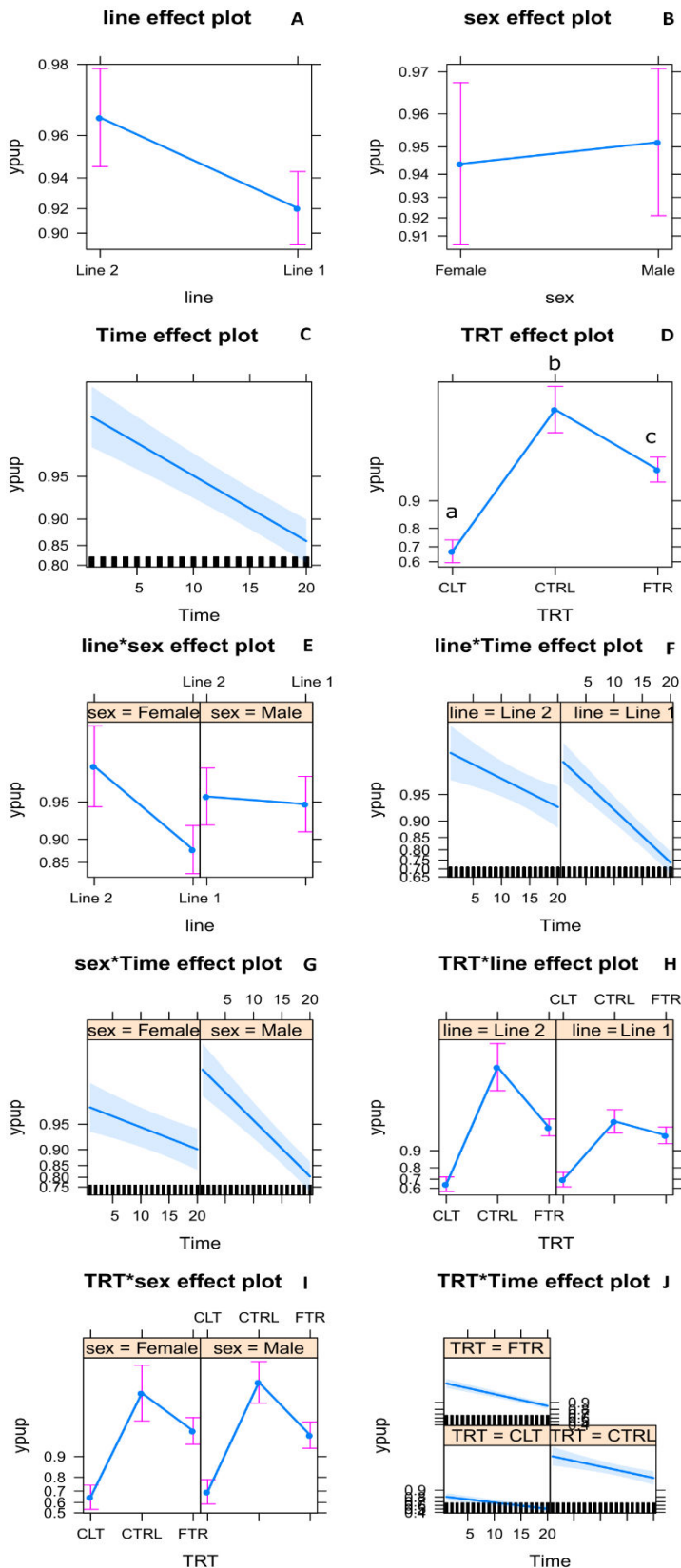


Figure S9: Effect plots from GLMM: Post storage survival of pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line and their interactions. Different letters on Fig. S9D correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

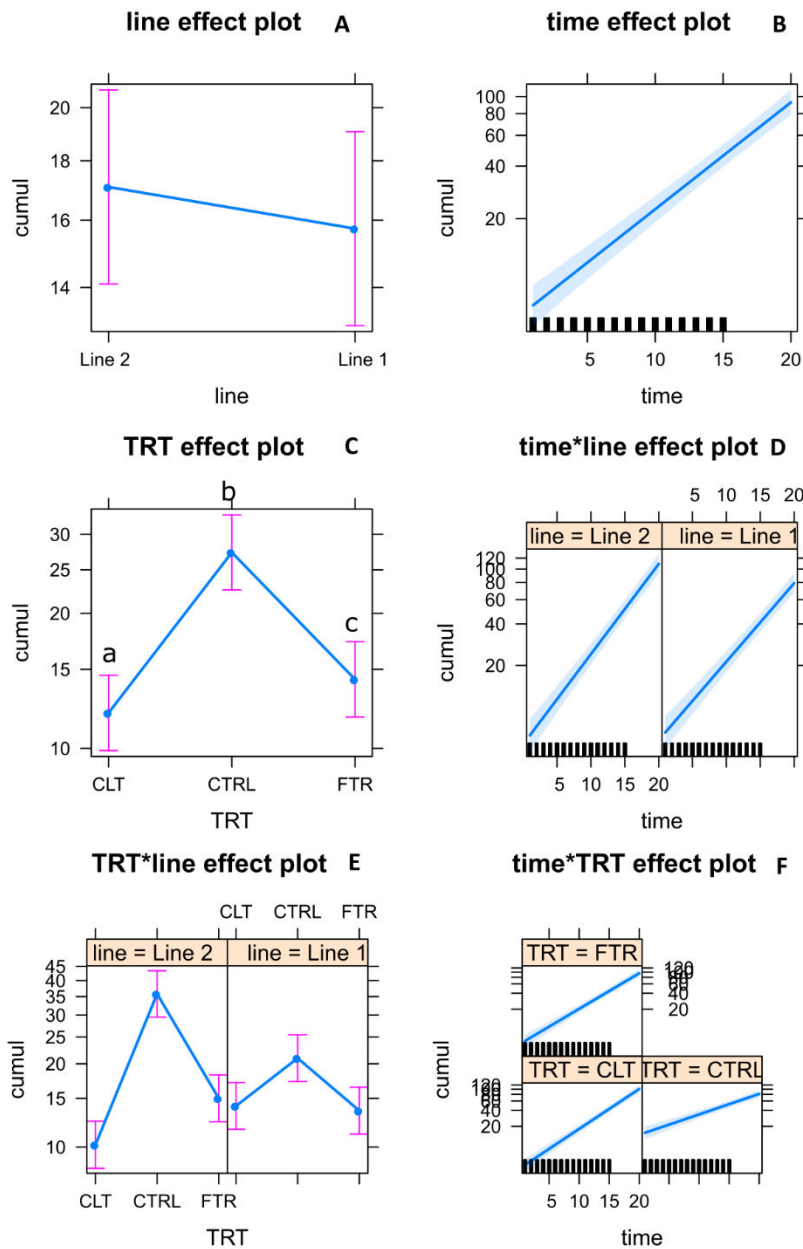


Figure S10: Effect plots from GLMM: Female fecundity of adults. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), line and their interactions. Different letters on Fig. S10C correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

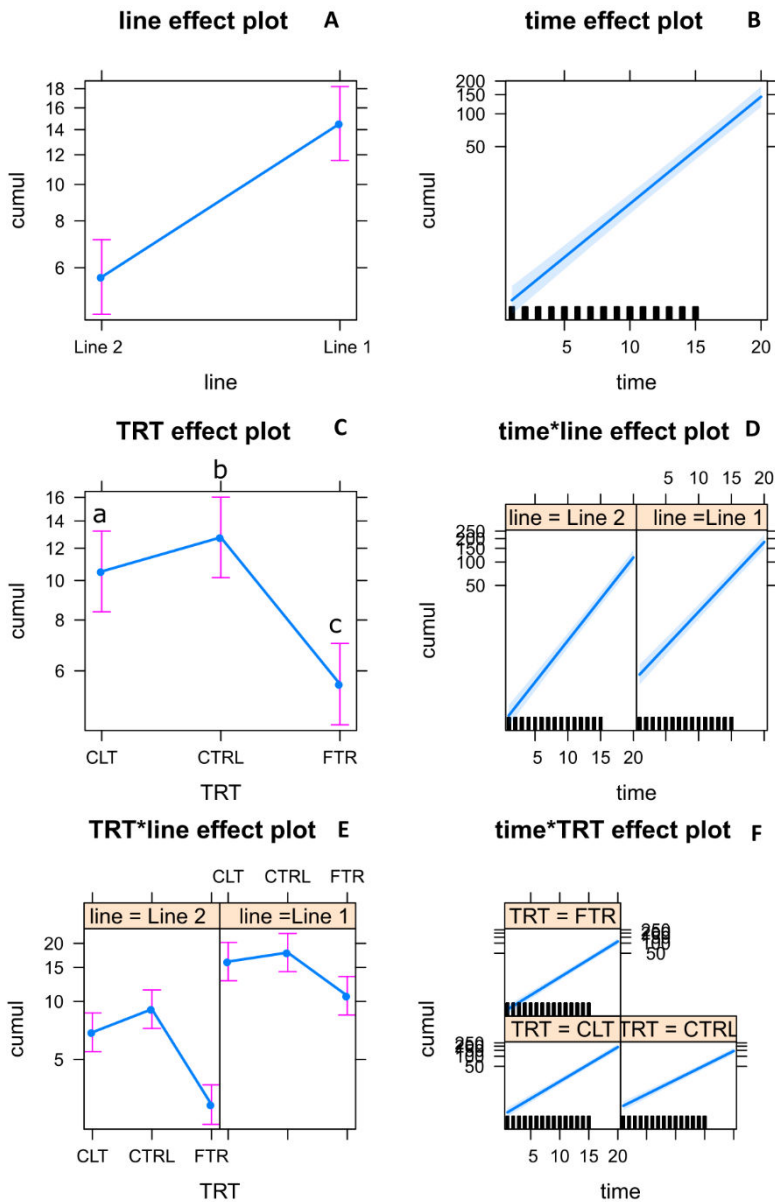
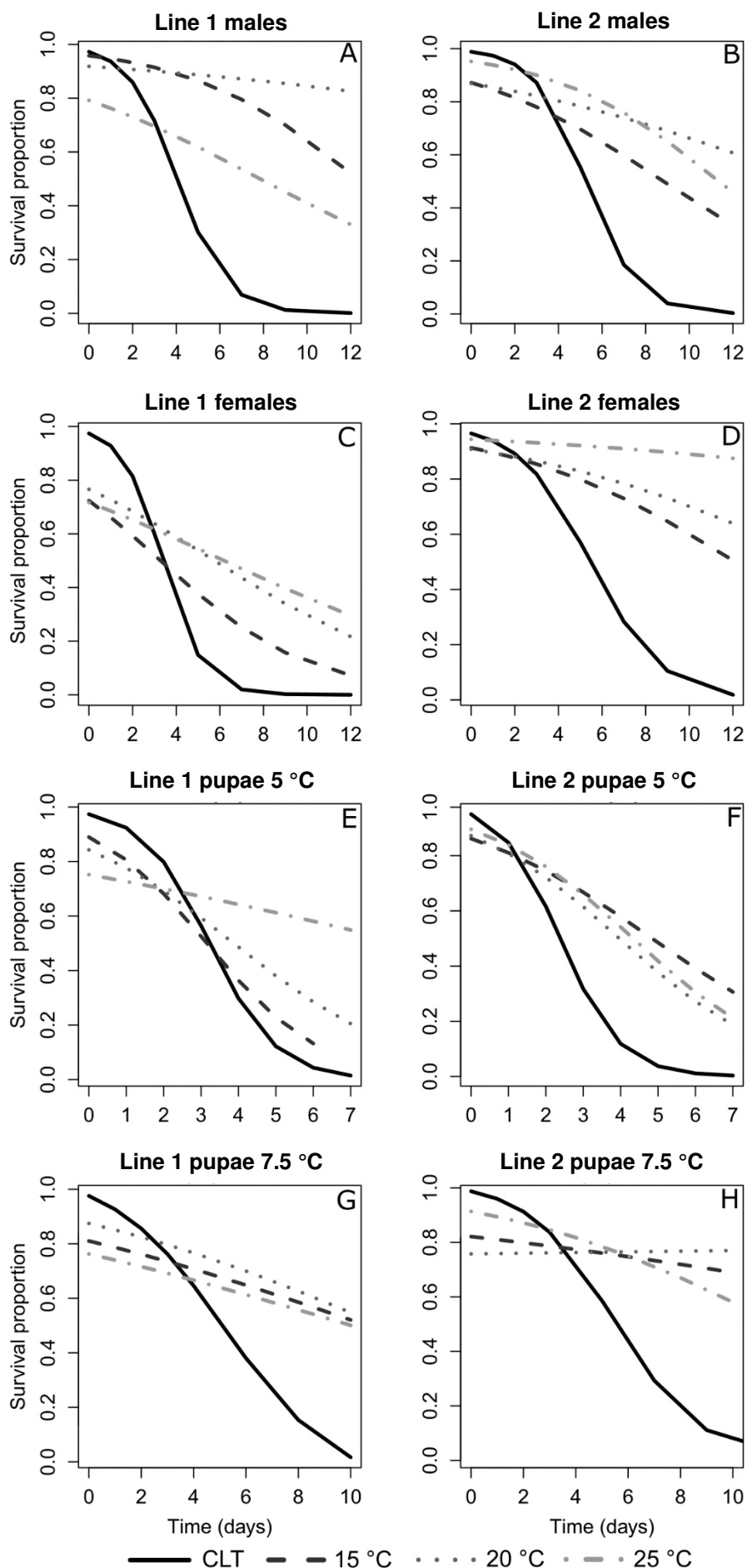


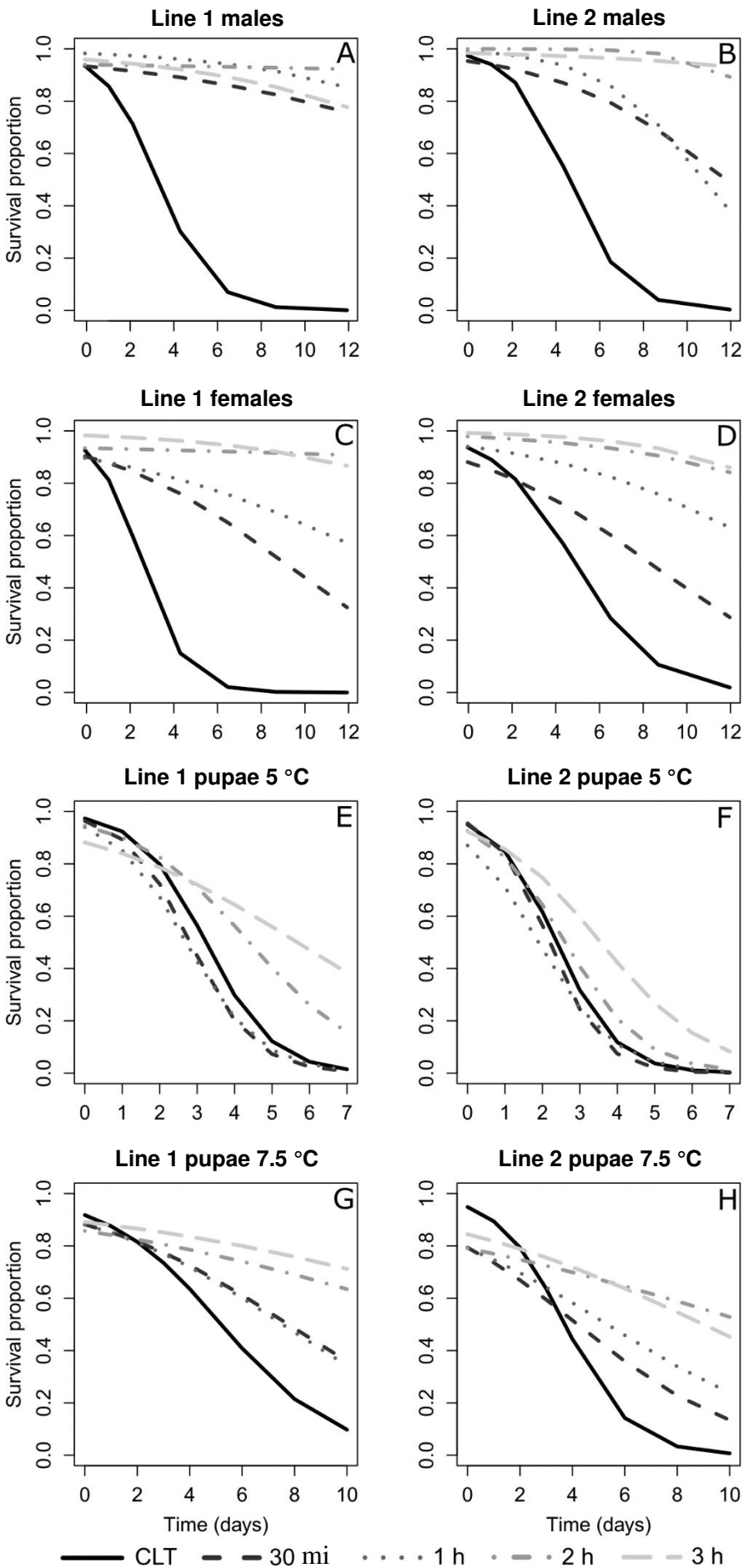
Figure S11: Effect plots from GLMM: Female fecundity of pupae. The plots show the conditional coefficients (“marginal effects”) of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), line and their interactions. Different letters on Fig. S11C correspond to statistical differences between treatments (least-squares means, $p < 0.05$).

Figures S12: Results from experiments 1 to 7 differentiate between lines 1 and 2. Each figure is associated with its caption. (Below:)



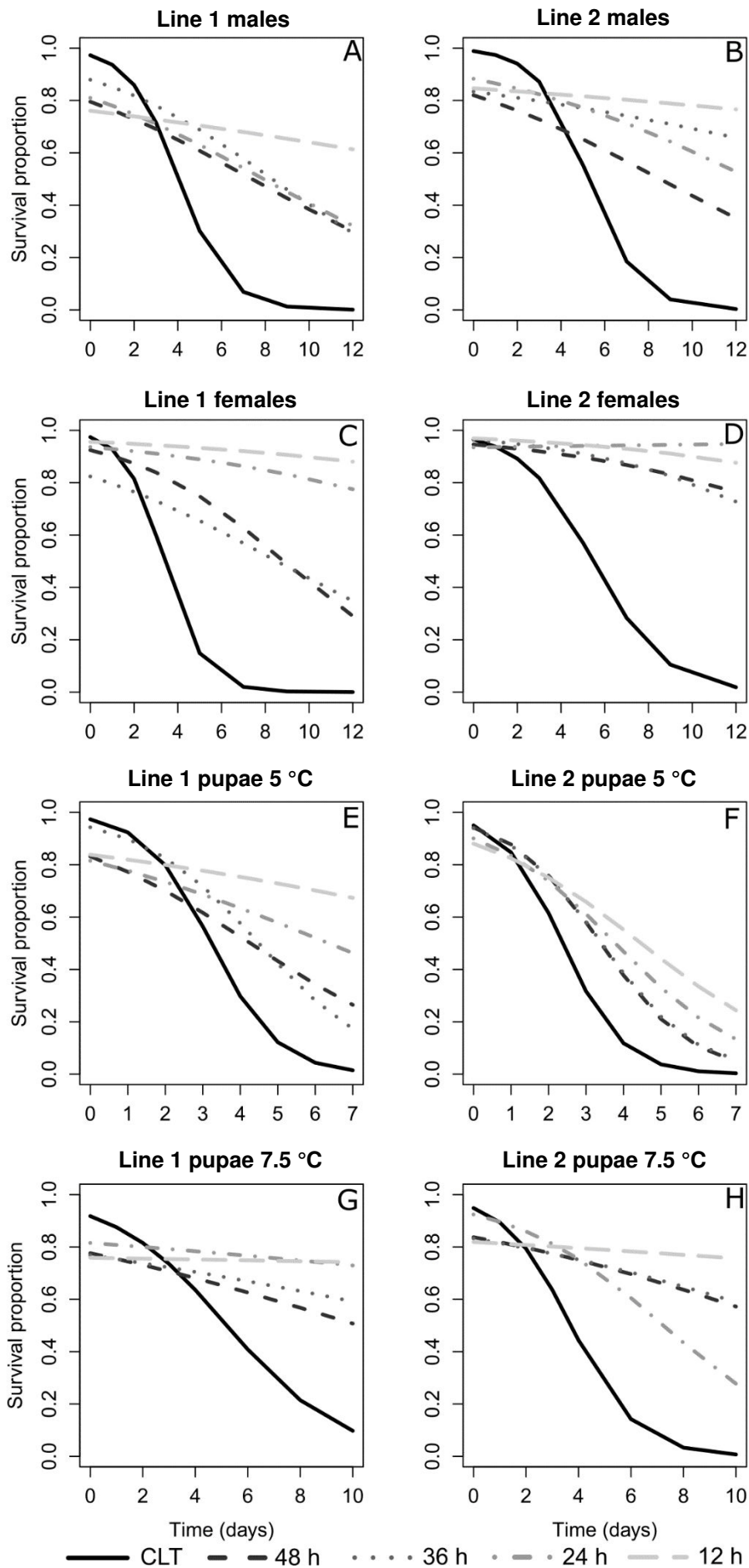
Exp. 1: Survival responses to changes in recovery temperature.

Solid black line: Insects subjected to constant cold exposure (CLT). Dashed grey lines: FTR where the cold exposure was interrupted by a daily recovery period of 2 h at 15, 20 or 25 °C. The lines correspond to model predictions (Binomial GLM, logit link function). A: Males from line 1; B: Males from line 2; C: Females from line 1; D: Females from line 2; E: Pupae from line 1 exposed to 5 °C; F: Pupae from line 2 exposed to 5 °C; G: Pupae from line 1 exposed to 7.5 °C; H: Pupae from line 2 exposed to 7.5 °C.

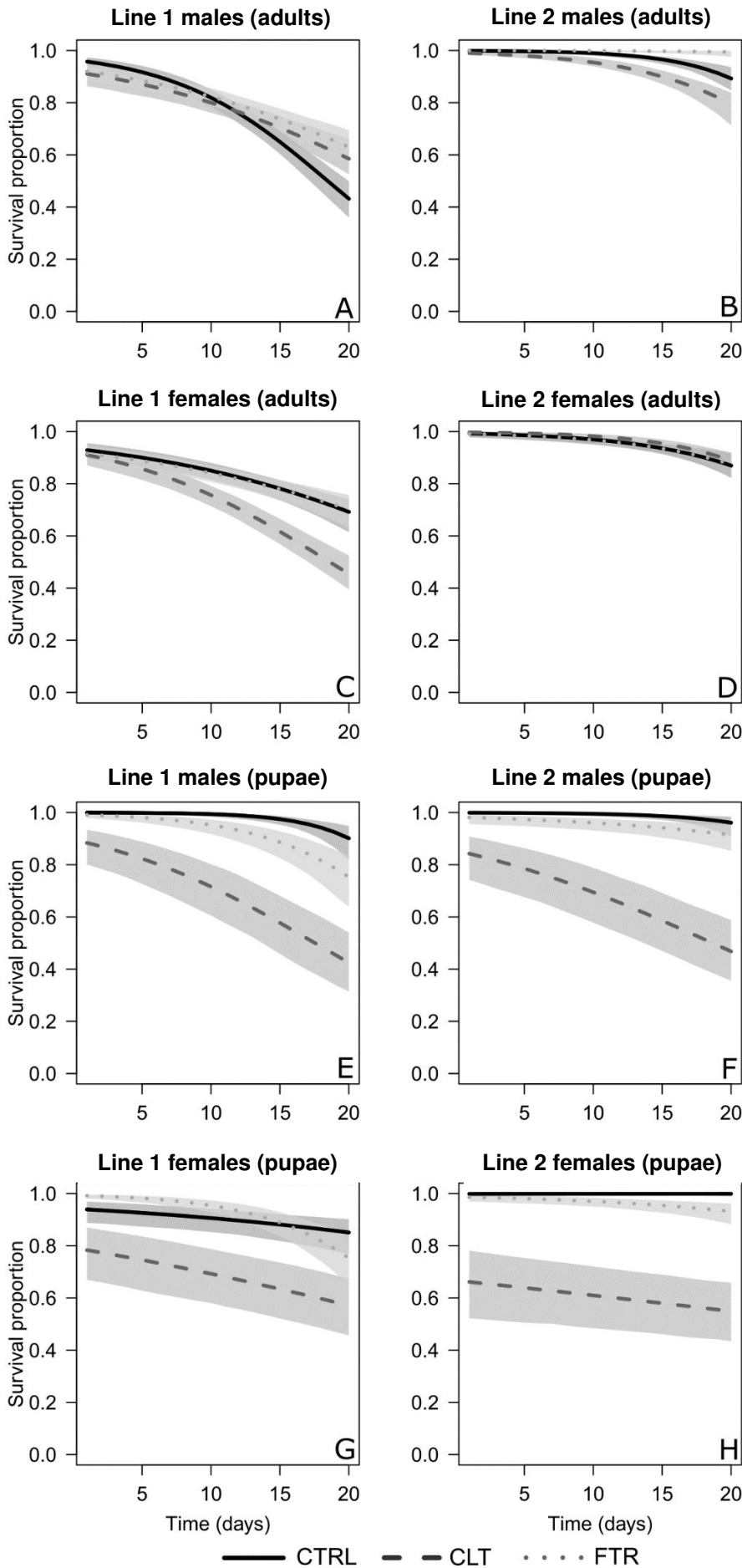


Exp. 2: Survival responses to changes in recovery duration.

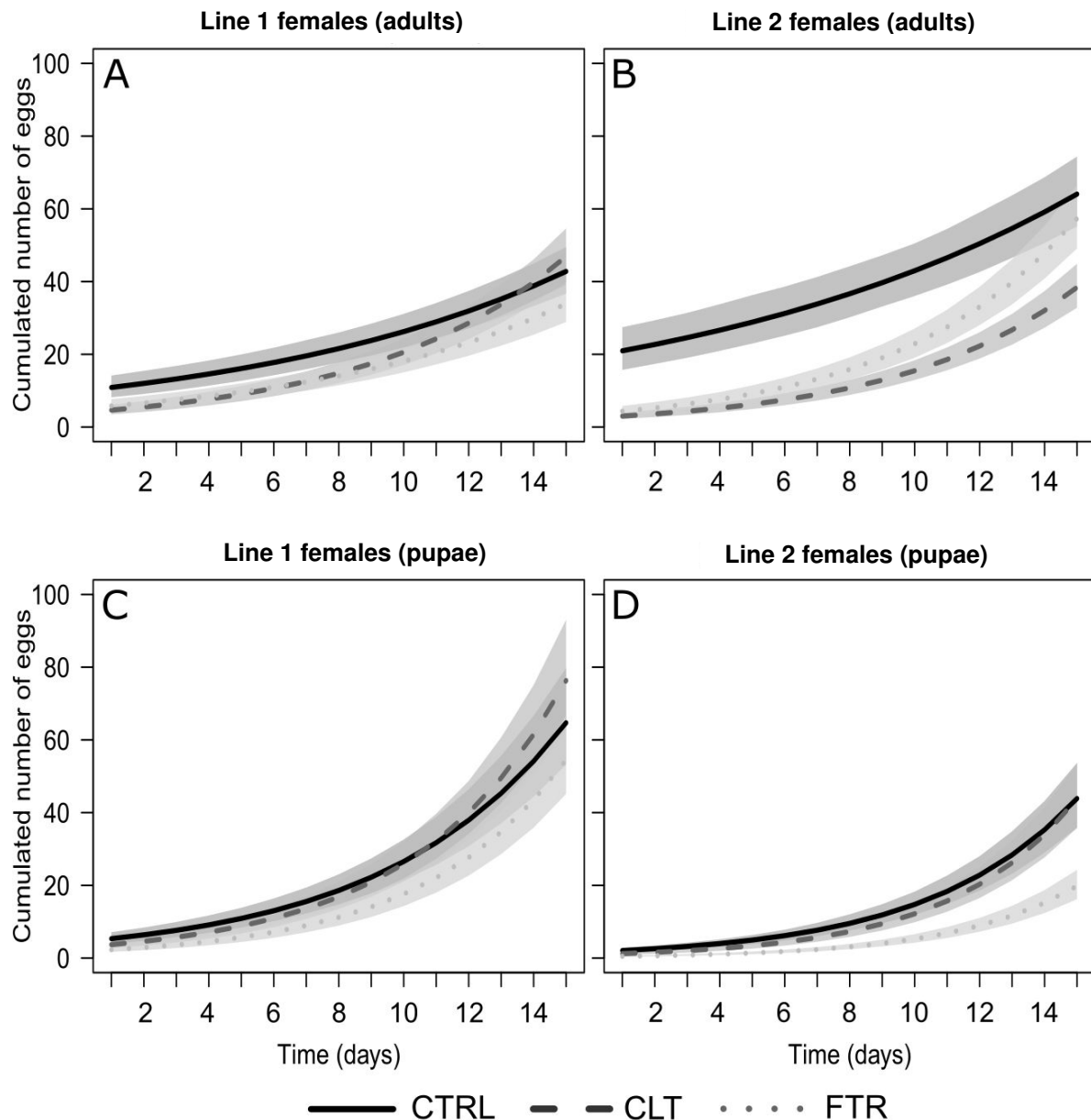
Solid black line: Insects subjected to constant cold exposure (CLT). Dashed grey lines: FTR where the cold exposure was interrupted by a daily recovery period at 20 °C for 30 min, 1, 2 or 3 h. The lines correspond to model predictions (Binomial GLM, logit link function). A: Males from line 1; B: Males from line 2; C: Females from line 1; D: Females from line 2; E: Pupae from line 1 exposed to 5 °C; F: Pupae from line 2 exposed to 5 °C; G: Pupae from line 1 exposed to 7.5 °C; H: Pupae from line 2 exposed to 7.5 °C.



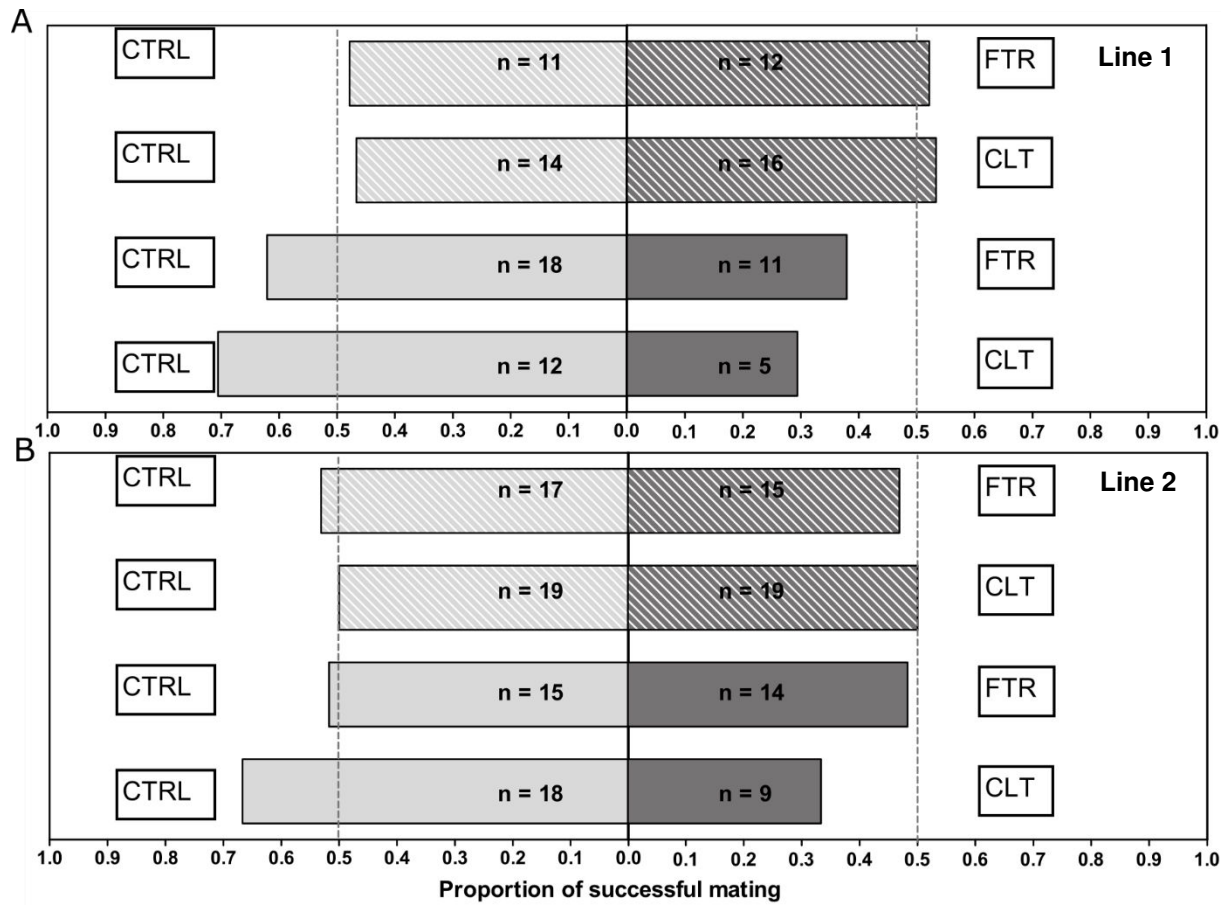
Exp. 3: Survival responses to changes in recovery frequency. Solid black line: Insects subjected to constant cold exposure (CLT). Dashed grey lines: FTR where the cold exposure was interrupted by a 3h-recovery period at 20 °C, applied every 48 h, 36 h, 24 h or 12 h. The lines correspond to model predictions (Binomial GLM, logit link function). A: Males from line 1; B: Males from line 2; C: Females from line 1; D: Females from line 2; E: Pupae from line 1 exposed to 5 °C; F: Pupae from line 2 exposed to 5 °C; G: Pupae from line 1 exposed to 7.5 °C; H: Pupae from line 2 exposed to 7.5 °C.



Exp. 5: Survival at 25 °C subsequent to a short cold-exposure under different regimes. Solid black line: control (CTRL: 25 °C for 5 days); dashed grey line: CLT applied to adults (5 °C for 5 days) and to pupae (7.5 °C for 5 days); dotted grey line: FTR where the cold exposure was interrupted by breaks of 3 h at 20 °C every 12 h. FTR was applied to adults and pupae for 5 days. After exposure to CTRL, CLT and FTR for 5 days, survival was monitored for 20 days under standard conditions. The lines correspond to model predictions (Binomial GLMM, logit link function), and shaded areas to 95% interval confidence. A: Adult males from line 1; B: Adult males from line 2; C: Adult females from line 1; D: Adult females from line 2; E: Males from cold-exposed pupae (line 1); F: Males from cold-exposed pupae (line 2); G: Females from cold-exposed pupae (line 1); H: Females from cold-exposed pupae (line 2).



Exp. 6: 15-days cumulated fecundity subsequent to a short cold-exposure under different regimes. Solid black line: control (CTRL: 25 °C for 5 days); dashed grey line: CLT applied to adults (5 °C for 5 days) and to pupae (7.5 °C for 5 days); dotted grey line: FTR where the cold exposure was interrupted by breaks of 3 h at 20 °C every 12 h. FTR was applied to adults and pupae for 5 days. After exposure to CTRL, CLT and FTR for 5 days, the fecundity was monitored for 15 days under standard conditions. The lines correspond to model predictions (Poisson GLMM, log link function), and shaded areas to 95% interval confidence. A: Adult females from line 1; B: Adult females from line 2; C: Females from cold-exposed pupae (line 1); D: Females from cold-exposed pupae (line 2).



Exp. 7: Male competitiveness subsequent to a short cold-exposure under different regimes. A: Line 1; B: Line 2. Light-grey bars: control males (CTRL: 25 °C for 5 days); dark-grey bars: males exposed to CLT (5 or 7.5°C for 5 days in adults and pupae respectively) and males exposed to FTR for 5 days (cold interrupted by breaks of 3 h at 20 °C every 12 h). Full bars correspond to individuals exposed to thermal treatments at pupal stage, and dashed bars correspond to individuals exposed as adults. After exposure to CTRL, CLT and FTR for 5 days, male competitiveness was monitored in mating assays. Numbers of successful males are indicated inside their respective bars.

Table S1: Detailed statistics from GLMs and GLMMs. Datasets from variation of temperature, duration and frequency of the recovery period and long-term storage GLMs were used. Post storage survival and female fecundity datasets were analyzed with GLMMs. ***: < 0.001; **: < 0.01; *: < 0.05; NS: not significant. TRT stands for thermal treatment (i.e. for experiments 1 to 4: CLT vs FTR, and for experiments 5 and 6: CTRL vs FTR and CLT). The symbol “ / ” indicate parameters not considered in the experiments and the sign “ - ” is used for data not included in the statistical analysis. TRT: treatment (CLT: constant low temperature; FTR: fluctuating thermal regime; CTRL: control).

Experiment:	Exp. 1- Recovery temperature						Exp. 2- Recovery duration						Exp. 3- Recovery frequency						Exp. 4- Long term storage			Exp. 5- Post storage survival						Exp. 6- Females fecundity					
	Adults			Pupae			Adults			Pupae			Adults			Pupae			Adults			Pupae			Adults			Pupae					
Parameter:	χ^2	df	p.value	χ^2	df	p.value	χ^2	df	p.value	χ^2	df	p.value	χ^2	df	p.value	χ^2	df	p.value	χ^2	df	p.value	χ^2	df	p.value	χ^2	df	p.value	χ^2	df	p.value			
line	153.93	1	***	0.13	1	NS	21.73	1	***	131.3	1	***	134.52	1	***	40.53	1	***	/	516.14	1	***	0.06	1	NS	334.54	1	***	2432.04	1	***		
temperature	/			190.38	1	***	/			460.13	1	***	/			249.14	1	***	/	/			/		/	/			/				
sex	33.42	1	***	/			32.1	1	***	/			95.76	1	***	/			66.47	1	***	1.52	1	NS	1.82	1	NS	/					
time	1077.69	1	***	581.84	1	***	1102.96	1	***	1167.05	1	***	947.14	1	***	713.34	1	***	519.95	1	***	293.47	1	***	145.33	1	***	286.88	1	***	663.67	1	***
TRT	350.52	3	***	166.45	3	***	1486.87	4	***	221.24	4	***	647.22	4	***	254.67	4	***	303.67	1	***	32.6	2	***	962.22	2	***	2072.35	2	***	988.22	2	***
temp:line	/			4.94	1	**	/			8.11	1	**	/			36.52	1	***	/	/			/		/			/					
time:line	3.19	1	NS	1.56	1	NS	1.39	1	NS	10.01	1	**	9.99	1	**	36.5	1	***	/	14.93	1	***	13.81	1	***	14.81	1	***	91.36	1	***		
time:temp	/			108.38	1	***	/			300.59	1	***	/			171.4	1	***	/	/			/		/		/						
TRT:line	44.13	3	***	49.17	3	***	43.38	4	***	2.17	4	NS	12.39	4	*	25.74	4	***	/	1.65	2	NS	33.38	2	***	666.81	2	***	255.86	2	***		
TRT:temp	/			19.41	3	***	/			27.99	4	***	/			22.31	4	***	/	/			/		/		/						
time:TRT	408.1	3	***	221.14	3	***	291.13	4	***	135.6	4	***	505.21	4	***	237.39	4	***	0.41	1	NS	9.06	2	*	19.09	2	***	652.6	2	***	119.88	2	***
sex:Time	0.21	1	NS	/			1.07	1	NS	/			0.11	1	NS	/			17.89	1	***	2.09	1	NS	20.02	1	***	/					
TRT:sex	40.98	3	***	/			23.9	4	***	/			87.98	4	***	/			8.38	1	**	6.39	2	*	25.88	2	***	/					
Line:sex	110.65	1	***	/			3.73	1	NS	/			11.92	1	***	/			/	0.2	1	NS	0.79	1	NS	/							
time:temp:line	/			0.95	1	NS	/			5.19	1	*	/			8.96	1	**	/	/			/		/		/						
TRT:temp:line	/			17.66	3	***	/			27.1	4	***	/			-			/	/			/		/		/						
time:TRT:line	8.91	3	*	24.09	3	**	22.62	4	***	-			6.64	4	NS	14.27	4	**	/	-			6.88	2	*	112.48	2	***	-				
time:TRT:temp	/			17.22	3	***	/			-			/			25.86	4	***	/	/			/		/		/						
TRT:Line:sex	23.66	3	***	/			21.11	4	***	/			10.89	4	*	/			/	67.48	2	***	17.54	2	***	/							
TRT:sex:Time	8.79	3	*	/			8.12	4	NS	/			7.54	4	NS	/			/	16.61	2	***	13.98	2	***	/							
time:sex:line	8.03	1	**	/			8.71	1	**	/			0.8	1	NS	/			/	-			-		/		/						

Annexe 4

Figures et tableaux supplémentaires de l'article 3 : Cold Acclimation Favors Metabolic Stability in *Drosophila suzukii*

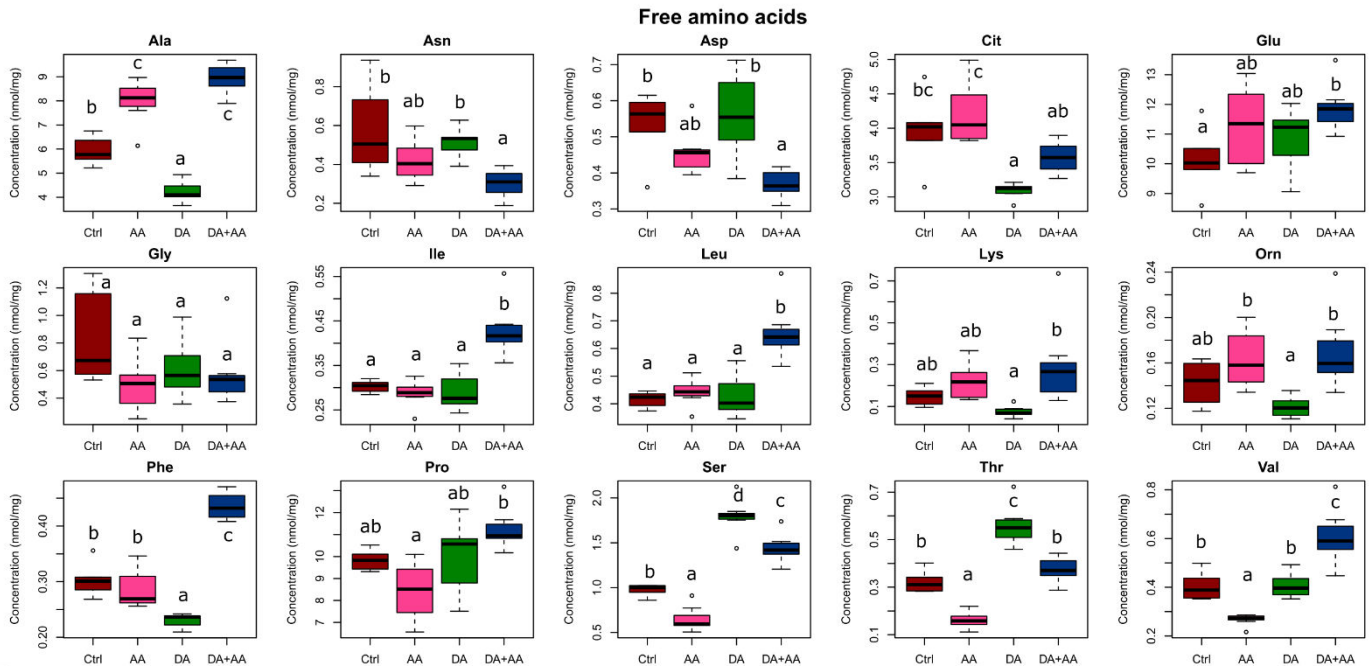


Figure S1: Concentrations of each free amino acid after the acclimation period. Boxplot sharing a same letter are not significantly different (p .value < 0.05; Tukey test). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

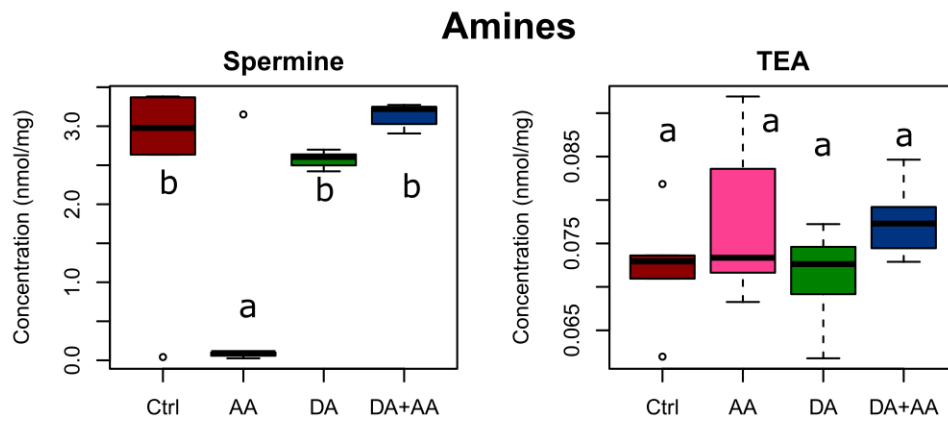


Figure S2: Concentrations of each amine after the acclimation period. Boxplot sharing a same letter are not significantly different (p .value < 0.05; Tukey test). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

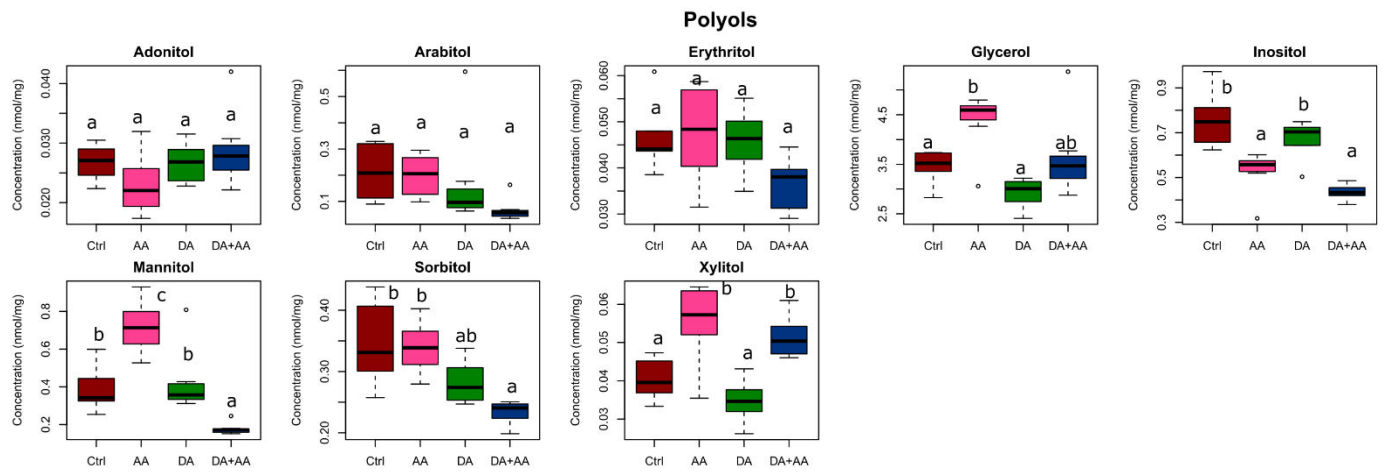


Figure S3: Concentrations of each polyol after the acclimation period. Boxplot sharing a same letter are not significantly different (p .value < 0.05; Tukey test). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

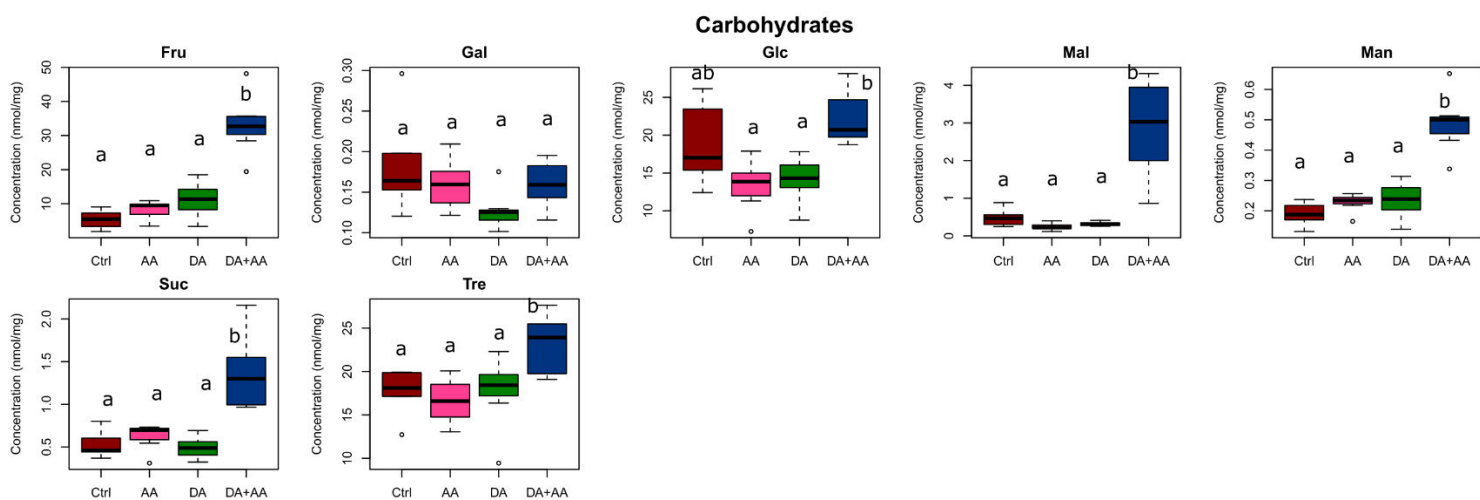


Figure S4: Concentrations of each carbohydrate after the acclimation period. Boxplot sharing a same letter are not significantly different (p .value < 0.05; Tukey test). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

TCA intermediates

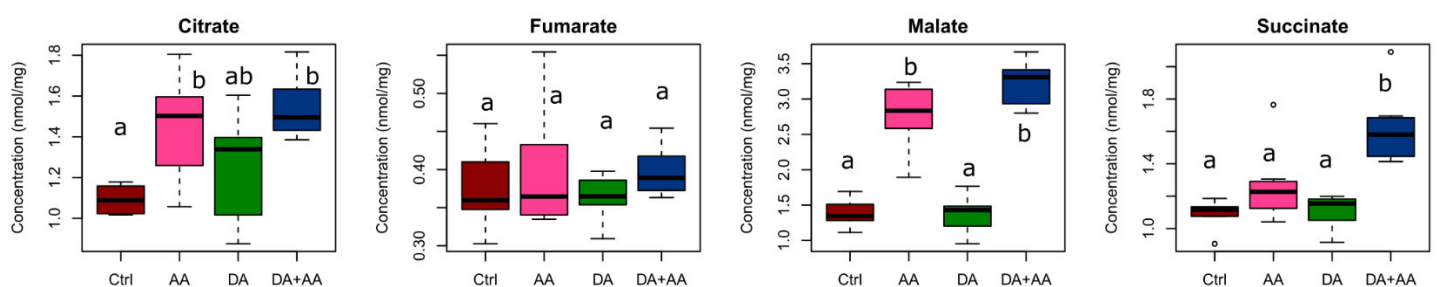


Figure S5: Concentrations of each TCA intermediate after the acclimation period. Boxplot sharing a same letter are not significantly different (p .value < 0.05; Tukey test). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

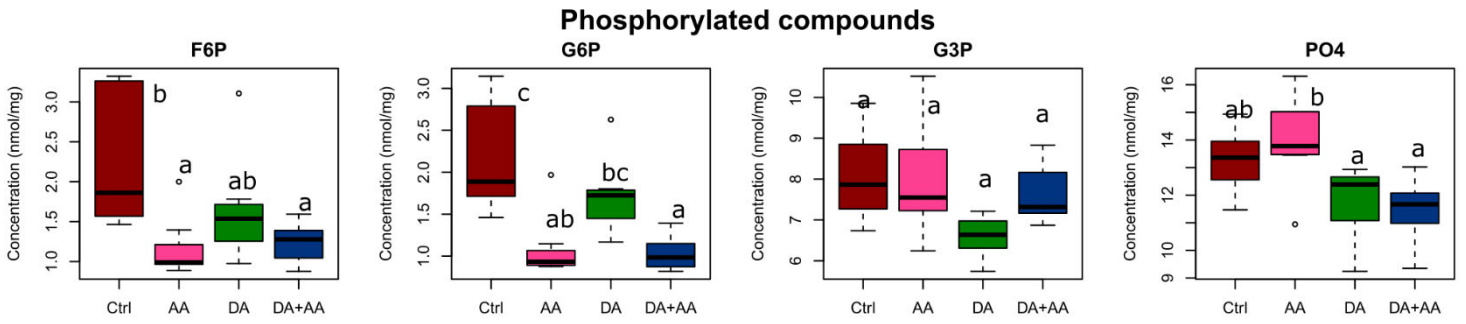


Figure S6: Concentrations of each phosphorylated compound after the acclimation period. Boxplot sharing a same letter are not significantly different (p .value < 0.05; Tukey test). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

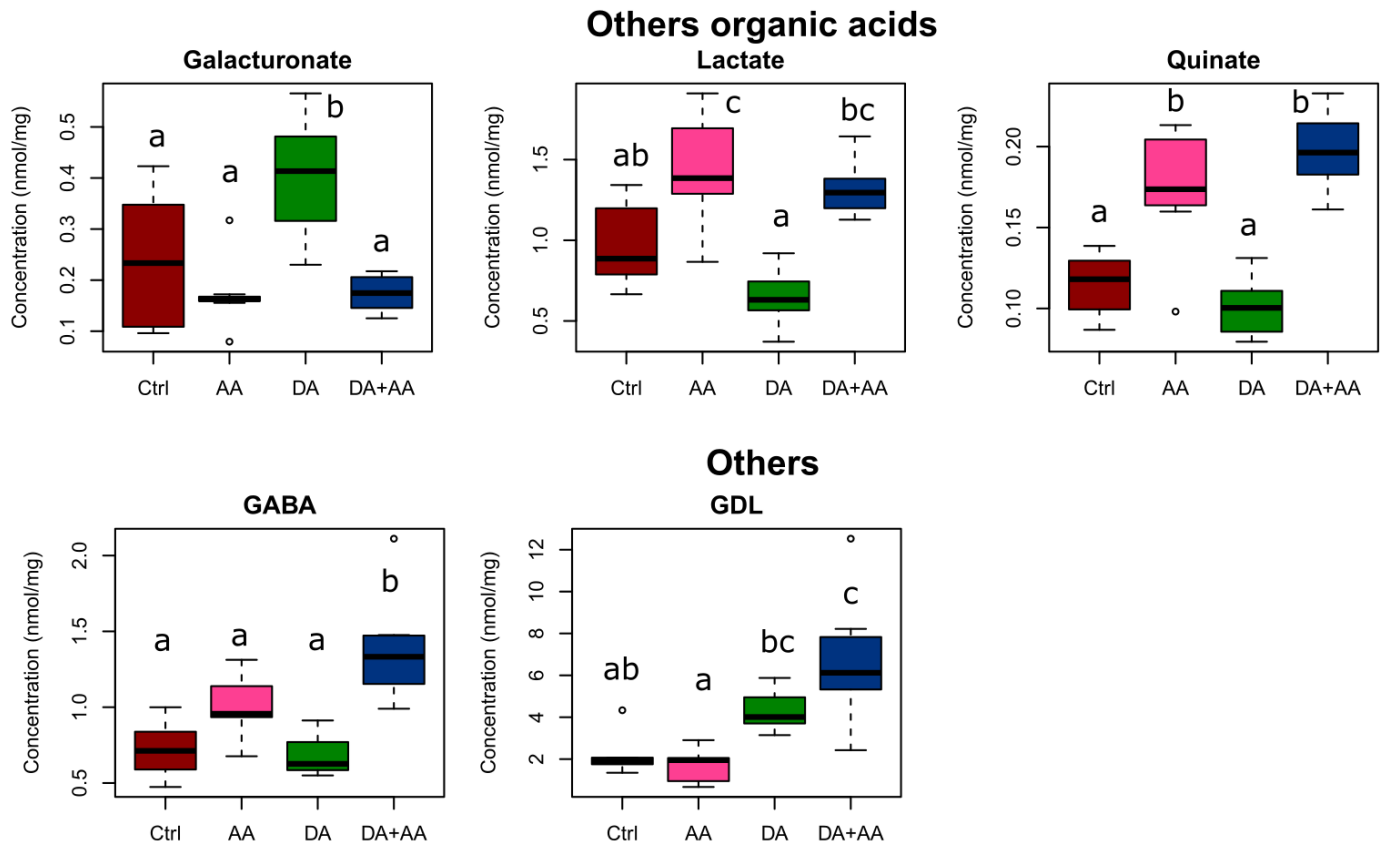


Figure S7: Concentrations of each other organic acids and other metabolites after the acclimation period. Boxplot sharing a same letter are not significantly different (p .value < 0.05; Tukey test). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

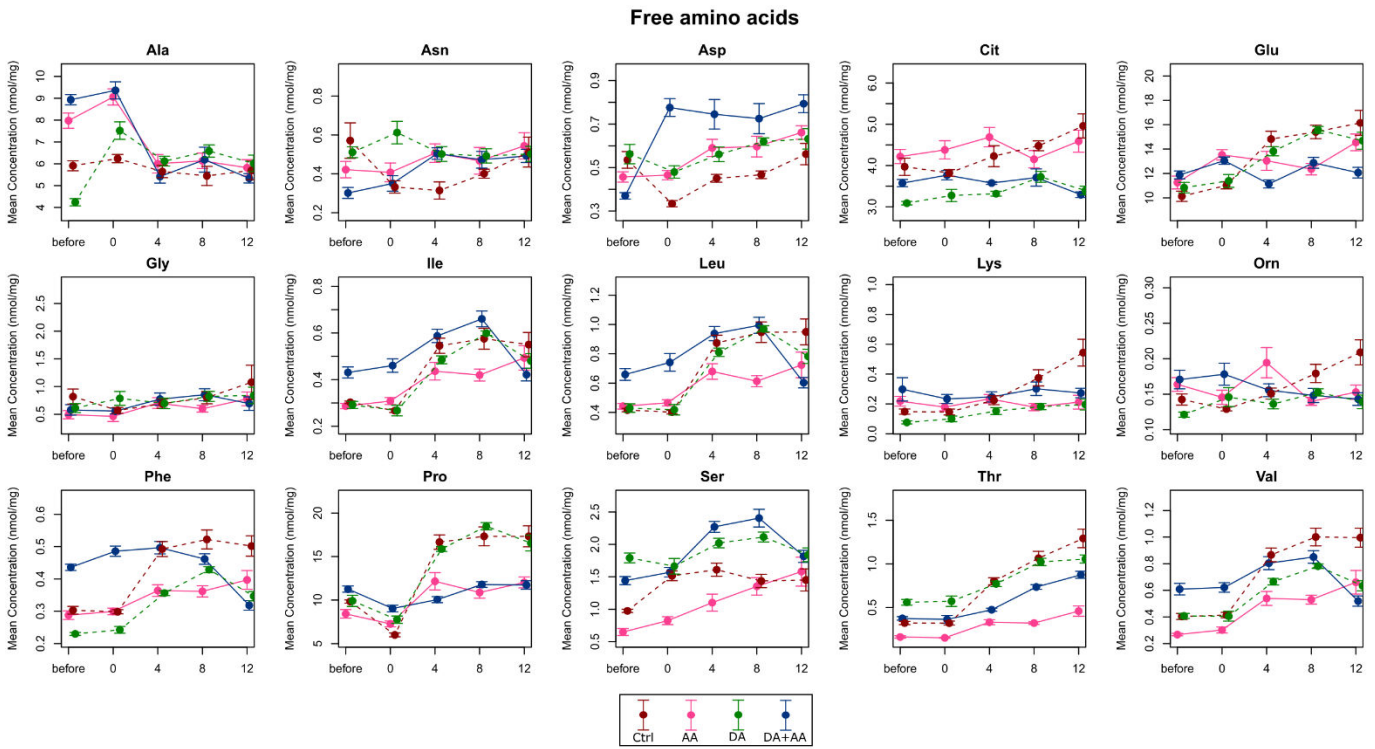


Figure S8: Temporal changes in concentrations of each free amino acid during the recovery from a cold stress at -5°C during 100 min (before, 0 h, 4 h, 8 h and 12 h after the cold stress). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

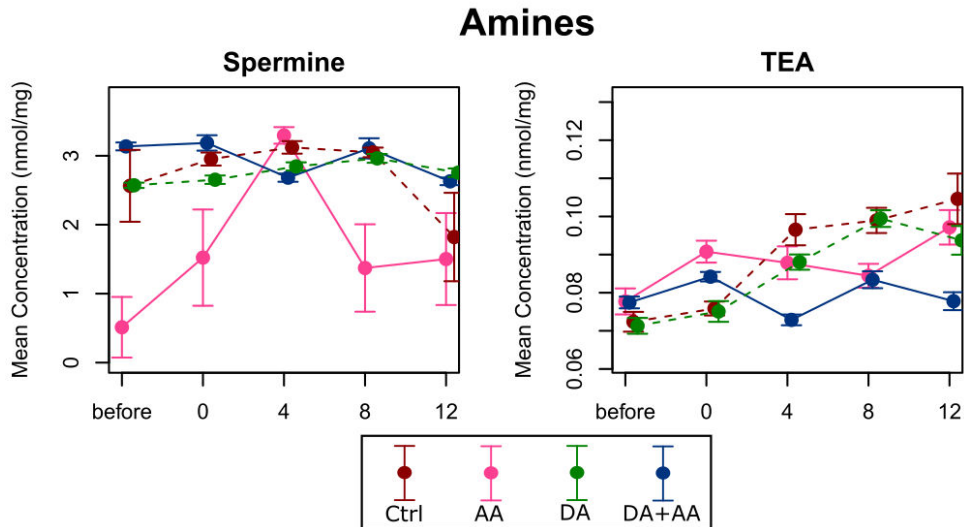


Figure S9: Temporal changes in concentrations of each amine during the recovery from a cold stress at -5°C during 100 min (before, 0 h, 4 h, 8 h and 12 h after the cold stress). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

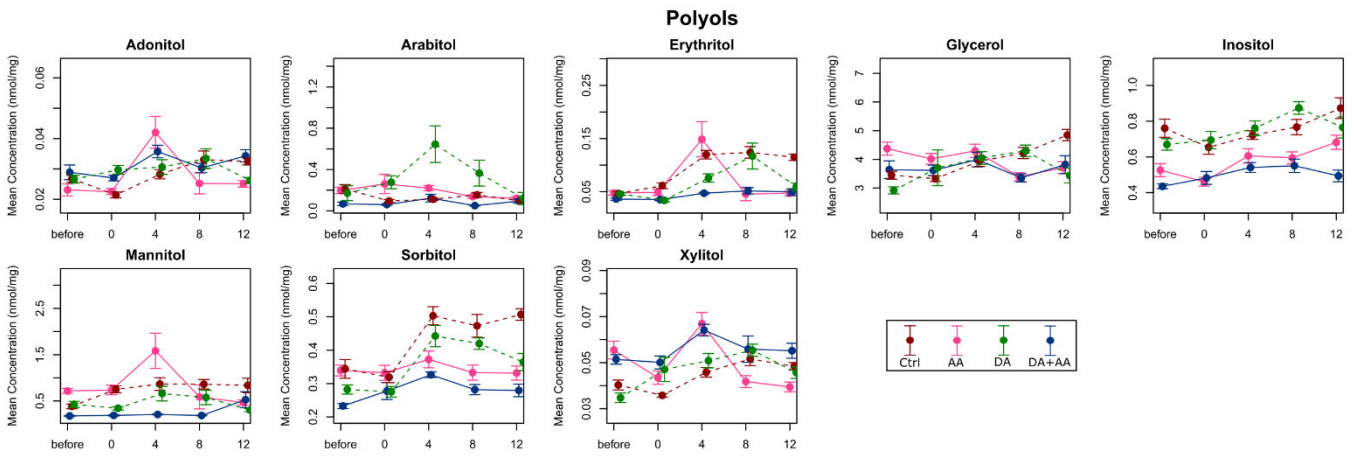


Figure S10: Temporal changes in concentrations of each polyol during the recovery from a cold stress at -5 °C during 100 min (before, 0 h, 4 h, 8 h and 12 h after the cold stress). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

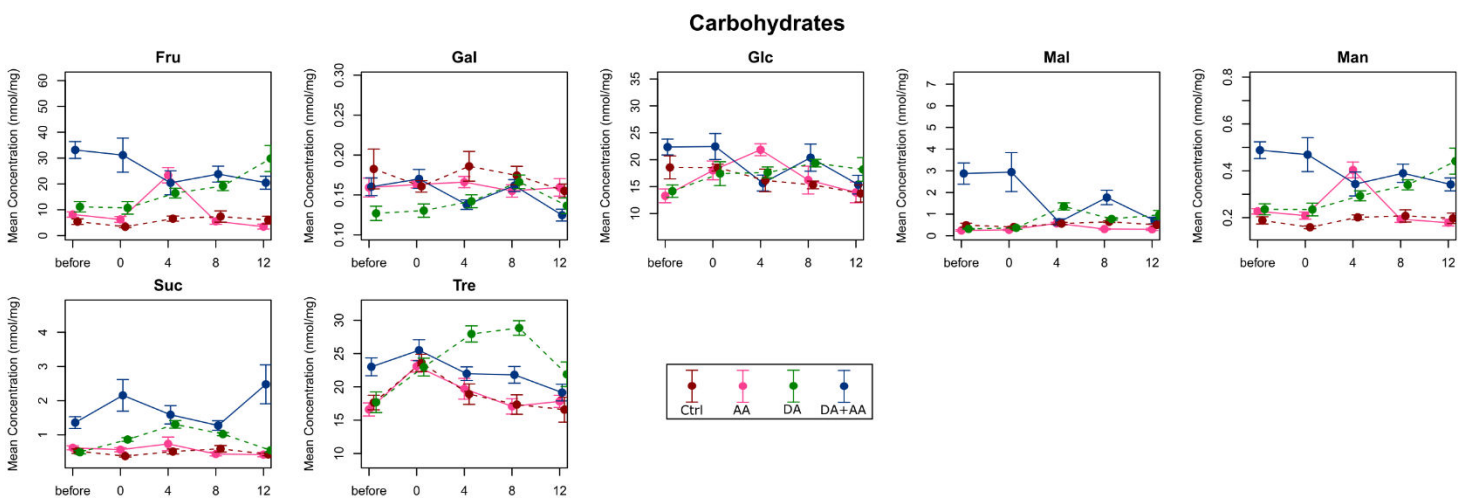


Figure S11: Temporal changes in concentrations of each carbohydrate during the recovery from a cold stress at -5 °C during 100 min (before, 0 h, 4 h, 8 h and 12 h after the cold stress). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

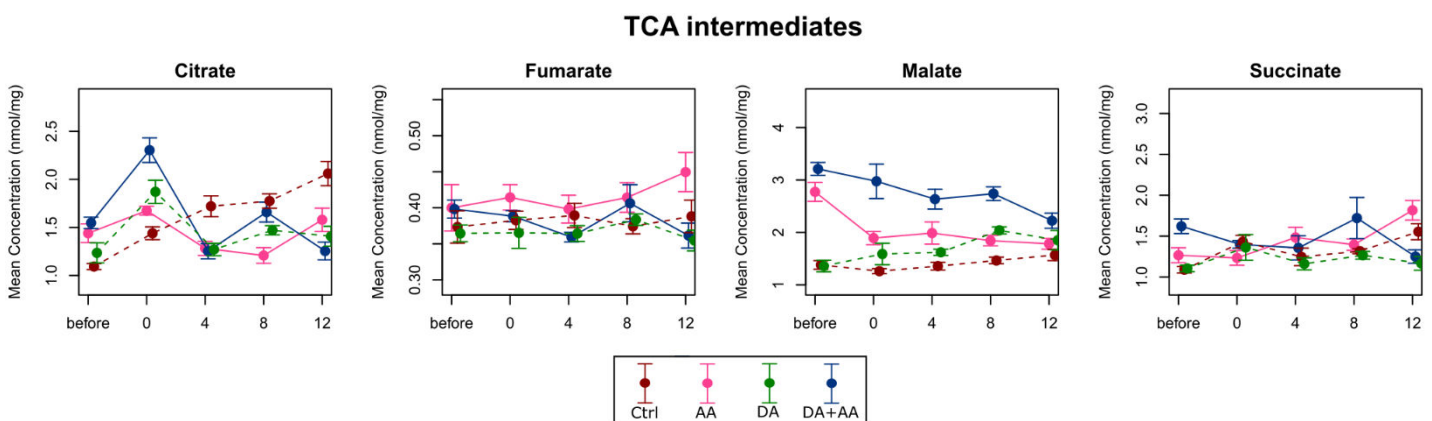


Figure S12: Temporal changes in concentrations of each TCA intermediate during the recovery from a cold stress at -5 °C during 100 min (before, 0 h, 4 h, 8 h and 12 h after the cold stress). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

Phosphorylated compounds

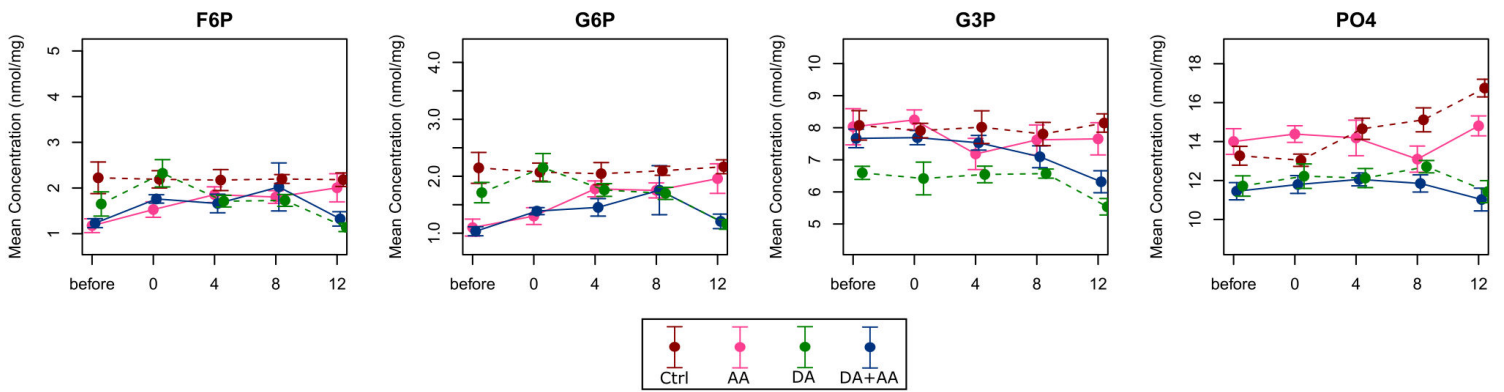


Figure S13: Temporal changes in concentrations of each phosphorylated compound during the recovery from a cold stress at -5°C during 100 min (before, 0 h, 4 h, 8 h and 12 h after the cold stress). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

Others organic acids

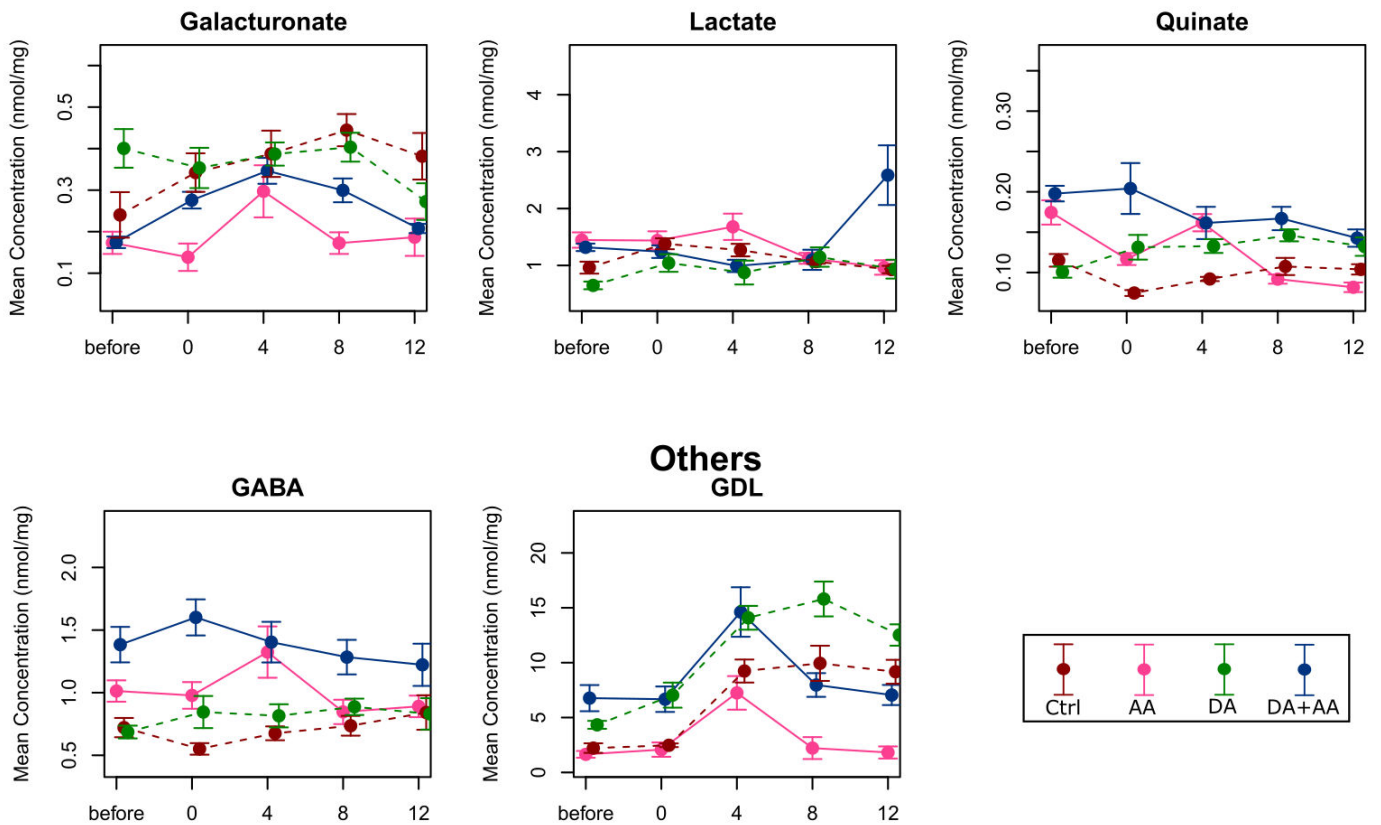
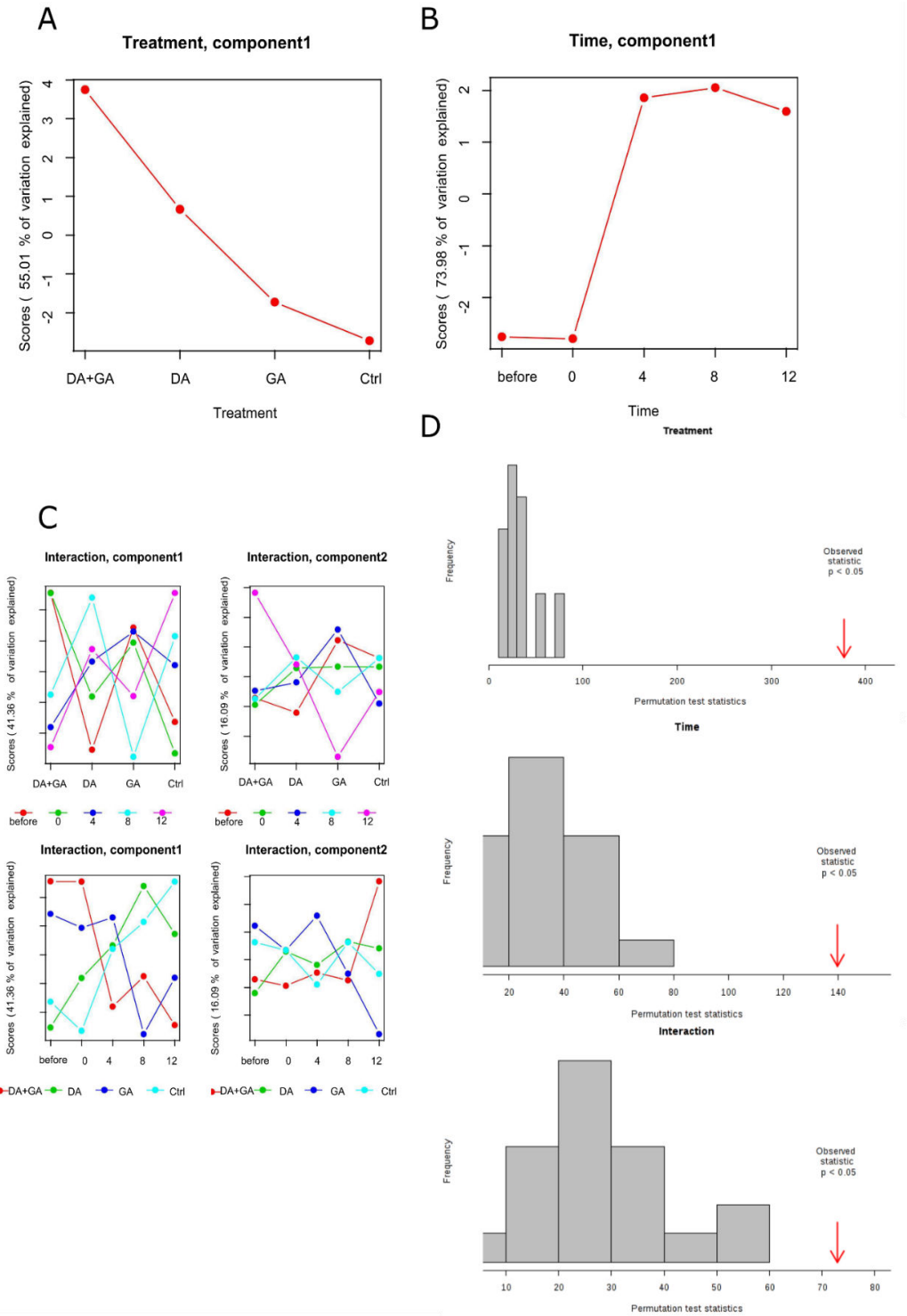


Figure S14: Temporal changes in concentrations of each other organic acids, and other metabolites during the recovery from a cold stress at -5°C during 100 min (before, 0 h, 4 h, 8 h and 12 h after the cold stress). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

Figure S15: ANOVA - Simultaneous Component Analysis (ASCA),

Metabolomic Data Analysis with MetaboAnalyst 4.0. ASCA is a multivariate extension of univariate ANOVA approach. It is designed to identify the major patterns associated with each factor. This implementation supports ASCA model for two factors with one interaction effect. The algorithm first partitions the overall data variance (X) into individual variances induced by each factor (A and B), as well as by the interactions (AB). The formula is shown below with (E) indicates the residual Errors: $X = A + B + AB + E$; $A = \text{Treatment (DA+AA, DA, AA, Ctrl)}$; $B = \text{Time}$. The SCA part applies PCA to A, B, AB to summarize major variations in each partition. Users then detect the major pattern by visualizing the PCA scores plot. MetaboAnalyst also provides model validation to test the significance of the effects associated with main effects. It is based on the Manly's unrestricted permutation of observation then calculate the permuted variation associated with each factor. Major patterns associated with factor A (treatment) (A). Major patterns associated with factor B (Time) (B). Major patterns associated with inzteraction between the two factors (C). Results of model validations through permutations (D). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.



summarize major variations in each partition. Users then detect the major pattern by visualizing the PCA scores plot. MetaboAnalyst also provides model validation to test the significance of the effects associated with main effects. It is based on the Manly's unrestricted permutation of observation then calculate the permuted variation associated with each factor. Major patterns associated with factor A (treatment) (A). Major patterns associated with factor B (Time) (B). Major patterns associated with inzteraction between the two factors (C). Results of model validations through permutations (D). Ctrl: Control flies; DA: developmental acclimation; AA: adult acclimation; DA+AA: combined acclimation.

Table S1: Metabolites identified with GC-MS and their respective abbreviation. Metabolites are grouped by biochemical families.

Free amino acids	Amines	Polyols	Carbohydrates	TCA intermediates	Phosphorylated compounds	Others organic acids	Others
Alanine (Ala)	Spermine	Adonitol	Fructose (Fru)	Citrate	Fructose-6-phosphate (F6P)	Galacturonate	γ -Aminobutyric acid (GABA)
Asparagine (Asn)	Triethanolamine (TEA)	Arabitol	Galactose (Gal)	Fumarate	Glucose-6-phosphate (G6P)	Lactate	Gluconolactone (GDL)
Aspartate (Asp)		Erythritol	Glucose (Glc)	Malate	Glycerol-3-phosphate (G3P)	Quinate	
Citrulline (Cit)		Glycerol	Maltose (Mal)	Succinate	Phosphoric acid (PO ₄)		
Glutamate (Glu)		Inositol	Mannose (Man)				
Glycine (Gly)		Mannitol	Saccharose (Suc)				
Isoleucine (Ile)		Sorbitol	Trehalose (Tre)				
Leucine (Leu)		Xylitol					
Lysine (Lys)							
Ornithine (Orn)							
Phenylalanine (Phe)							
Proline (Pro)							
Serine (Ser)							
Threonine (Thr)							
Valine (Val)							

Metabolites	Parameter	χ^2	df	p.value	Metabolites	Interaction	χ^2	df	p.value
Ala	TRT	50.46	3	< 0.001***	Sorbitol	TRT	122.67	3	< 0.001***
	Time	125.86	4	< 0.001***		Time	83.08	4	< 0.001***
	TRT x Time	124.26	12	< 0.001***		TRT x Time	49.10	12	< 0.001***
Asn	TRT	15.02	3	< 0.01**	Xylitol	TRT	33.67	3	< 0.001***
	Time	7.23	4	0.12		Time	43.61	4	< 0.001***
	TRT x Time	37.98	12	< 0.001***		TRT x Time	63.80	12	< 0.001***
Asp	TRT	78.93	3	< 0.001***	Fru	TRT	150.85	3	< 0.001***
	Time	58.16	4	< 0.001***		Time	3.98	4	0.41
	TRT x Time	77.62	12	< 0.001***		TRT x Time	78.83	12	< 0.001***
Cit	TRT	137.82	3	< 0.001***	Gal	TRT	23.16	3	< 0.001***
	Time	11.14	4	< 0.05*		Time	7.84	4	0.10
	TRT x Time	35.78	12	< 0.001***		TRT x Time	20.50	12	0.06
Glu	TRT	17.69	3	< 0.001***	Glc	TRT	8.26	3	< 0.05*
	Time	100.73	4	< 0.001***		Time	10.44	4	< 0.05*
	TRT x Time	89.66	12	< 0.001***		TRT x Time	32.63	12	< 0.01**
Gly	TRT	6.48	3	0.09	Mal	TRT	93.93	3	< 0.001***
	Time	12.01	4	< 0.05*		Time	5.99	4	0.20
	TRT x Time	9.80	12	0.63		TRT x Time	83.06	12	< 0.001***
Ile	TRT	46.45	3	< 0.001***	Man	TRT	127.37	3	< 0.001***
	Time	218.88	4	< 0.001***		Time	3.78	4	0.44
	TRT x Time	59.85	12	< 0.001***		TRT x Time	79.26	12	< 0.001***
Leu	TRT	46.22	3	< 0.001***	Suc	TRT	137.91	3	< 0.001***
	Time	228.28	4	< 0.001***		Time	6.25	4	0.18
	TRT x Time	87.74	12	< 0.001***		TRT x Time	37.25	12	< 0.001***
Lys	TRT	42.65	3	< 0.001***	Tre	TRT	53.67	3	< 0.001***
	Time	31.95	4	< 0.001***		Time	42.00	4	< 0.001***
	TRT x Time	49.62	12	< 0.001***		TRT x Time	49.73	12	< 0.001***
Orn	TRT	14.39	3	< 0.01**	Citrate	TRT	17.80	3	< 0.001***
	Time	3.53	4	0.47		Time	69.42	4	< 0.001***
	TRT x Time	57.34	12	< 0.001***		TRT x Time	129.64	12	< 0.001***
Phe	TRT	174.66	3	< 0.001***	Fumarate	TRT	19.32	3	< 0.001***
	Time	168.43	4	< 0.001***		Time	1.85	4	0.76
	TRT x Time	184.37	12	< 0.001***		TRT x Time	10.91	12	0.54
Pro	TRT	124.42	3	< 0.001***	Malate	TRT	228.65	3	< 0.001***
	Time	397.53	4	< 0.001***		Time	12.44	4	< 0.05*
	TRT x Time	145.52	12	< 0.001***		TRT x Time	57.98	12	< 0.001***
Ser	TRT	193.53	3	< 0.001***	Succinate	TRT	18.17	3	< 0.001***
	Time	88.24	4	< 0.001***		Time	7.40	4	0.12
	TRT x Time	50.74	12	< 0.001***		TRT x Time	39.81	12	< 0.001***
Thr	TRT	420.14	3	< 0.001***	F6P	TRT	21.44	3	< 0.001***
	Time	514.02	4	< 0.001***		Time	9.76	4	< 0.05*
	TRT x Time	113.16	12	< 0.001***		TRT x Time	22.33	12	< 0.05*
Val	TRT	115.62	3	< 0.001***	G6P	TRT	42.82	3	< 0.001***
	Time	237.29	4	< 0.001***		Time	8.35	4	0.08
	TRT x Time	99.91	12	< 0.001***		TRT x Time	32.56	12	< 0.01**
Spermine	TRT	46.36	3	< 0.001***	G3P	TRT	59.65	3	< 0.001***
	Time	16.17	4	< 0.01**		Time	8.98	4	0.06
	TRT x Time	34.11	12	< 0.001***		TRT x Time	12.57	12	0.40
TEA	TRT	31.47	3	< 0.001***	PO4	TRT	111.82	3	< 0.001***
	Time	88.25	4	< 0.001***		Time	6.32	4	0.18
	TRT x Time	78.67	12	< 0.001***		TRT x Time	35.68	12	< 0.001***
Adonitol	TRT	7.59	3	0.06	Galacturonate	TRT	64.34	3	< 0.001***
	Time	41.32	4	< 0.001***		Time	20.49	4	< 0.001***
	TRT x Time	49.35	12	< 0.001***		TRT x Time	21.67	12	< 0.05*
Arabitol	TRT	41.25	3	< 0.001***	Lactate	TRT	25.04	3	< 0.001***
	Time	15.72	4	< 0.01**		Time	6.09	4	0.19
	TRT x Time	36.77	12	< 0.001***		TRT x Time	67.36	12	< 0.001***
Erythritol	TRT	51.51	3	< 0.001***	Quinate	TRT	96.52	3	< 0.001***
	Time	73.80	4	< 0.001***		Time	14.14	4	< 0.01**
	TRT x Time	72.69	12	< 0.001***		TRT x Time	61.61	12	< 0.001***
Glycerol	TRT	6.29	3	0.10	GABA	TRT	94.81	3	< 2e-16***
	Time	9.65	4	< 0.05*		Time	2.74	4	0.60
	TRT x Time	48.55	12	< 0.001***		TRT x Time	18.36	12	0.11
Inositol	TRT	163.43	3	< 0.001***	GDL	TRT	128.68	3	< 0.001***
	Time	36.58	4	< 0.001***		Time	121.20	4	< 0.001***
	TRT x Time	21.74	12	< 0.05*		TRT x Time	53.84	12	< 0.001***
Mannitol	TRT	52.89	3	< 0.001***					
	Time	19.17	4	< 0.001***					
	TRT x Time	39.39	12	< 0.001***					

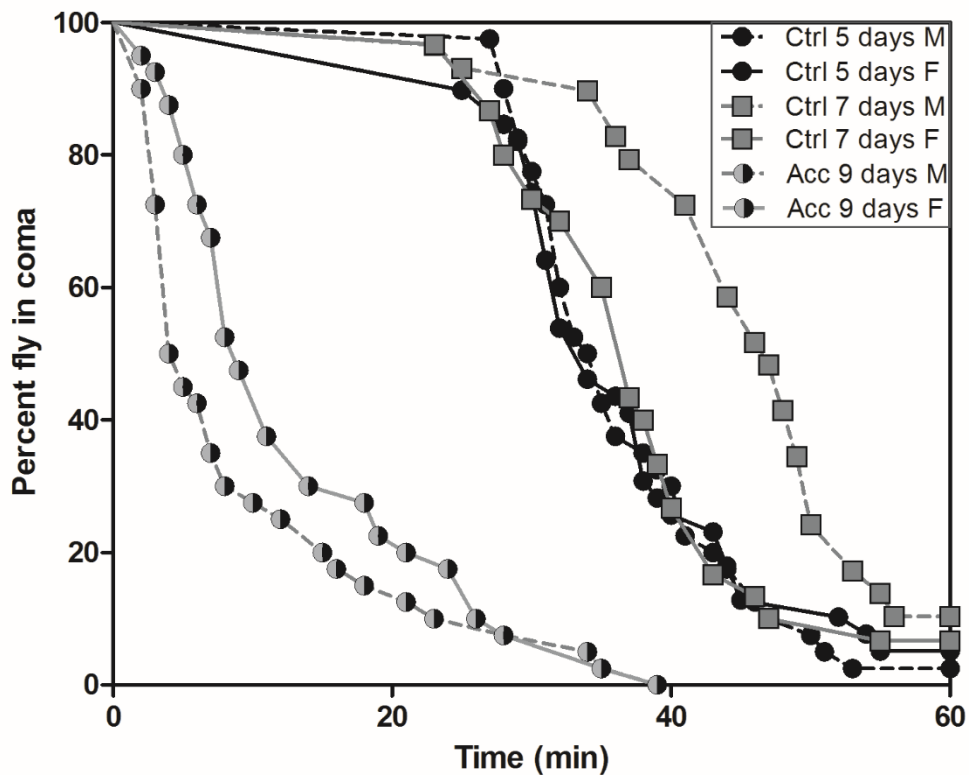
Table S2: Outcomes of GLMs on metabolites concentration during the recovery from the acute cold stress.

TRT: thermal treatment; Time: recovery time.

Annexe 5

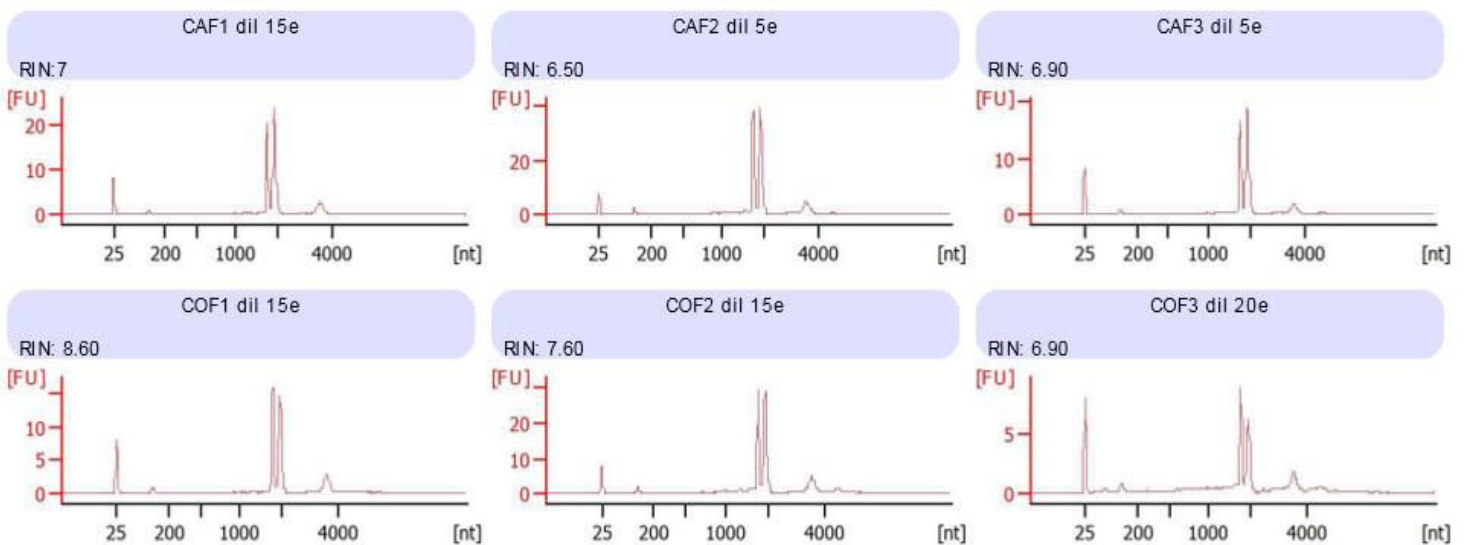
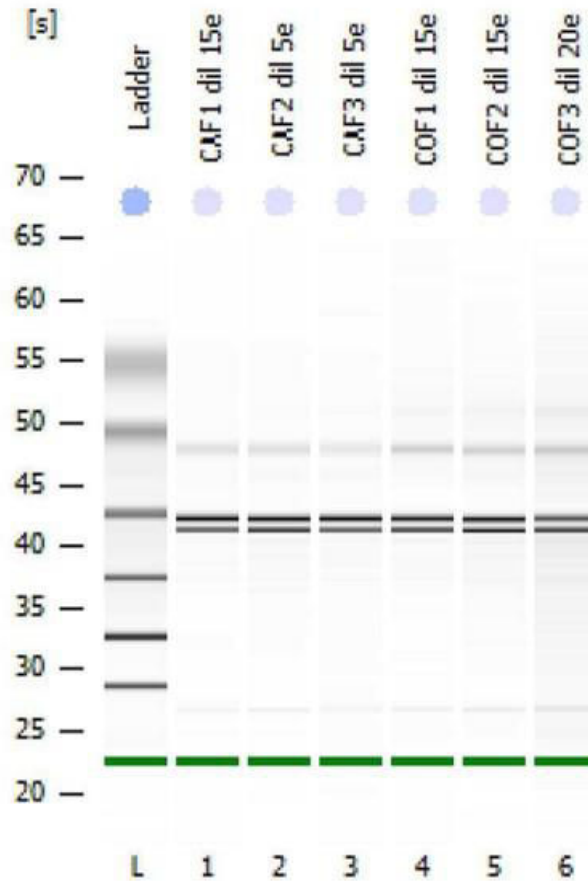
Figures supplémentaires de l'article 5 : Cold acclimation triggers major transcriptional changes in *Drosophila suzukii*

Supplementary Figure S1: Chill coma recovery time of control flies at two different age (5 and 7 days) and flies acclimated for 9 days. Flies have been submitted to 0°C for 12 h, and then their individual time to recover from coma was recorded at 25°C. Each point corresponds to the recovery time of one fly. Full lines: females, dotted lines: males.



Supplementary Figure S2: Bioanalyser report on RNA extract from Control (COF) and cold acclimated (CAF) samples (females of *D. sukii*). Cold acclimation consisted of 5 days old females exposed to 10°C during 9 days. (Agilent Bioanalyzer nanochip, Agilent, Palo Alto, CA).

Electrophoresis File Run Summary



Titre : Mécanismes physiologiques sous-jacents à la plasticité de la thermotolérance chez la drosophile invasive *Drosophila suzukii*

Mots clés : tolérance au froid ; températures fluctuantes ; acclimatation ; omiques ; lutte intégrée

Résumé : *Drosophila suzukii* est une drosophile invasive en Europe, Amérique du Nord et Amérique du Sud. Contrairement aux autres espèces de drosophiles, les femelles parasitent les fruits mûrs que les larves consomment, engendrant d'importants dégâts sur les cultures fruitières. Les stratégies mises en place par cette espèce pour tolérer les températures hivernales sous nos latitudes sont encore peu comprises. Par conséquent, l'objectif de ma thèse était d'acquérir des connaissances fondamentales sur la thermotolérance de cette espèce, en m'intéressant notamment à la plasticité de la tolérance au froid et aux mécanismes physiologiques sous-jacents à l'acclimatation. J'ai évalué la thermotolérance basale de *D. suzukii* en soumettant des adultes et des pupes à un large panel de températures (froides et chaudes). Ces expérimentations ont permis de confirmer que cette espèce était intolérante au froid et que des températures supérieures à 32°C impactaient grandement sa survie. Par la suite, j'ai évalué la plasticité de sa tolérance au froid. Mes travaux ont permis de confirmer que sa thermotolérance était effectivement plastique, puisque l'utilisation de

températures fluctuantes ou l'acclimatation permettaient de réduire sa mortalité lors d'expositions aux basses températures. L'acclimatation chez *D. suzukii* était corrélée à de nombreuses modifications physiologiques, telles que l'accumulation de cryoprotecteurs, un réajustement de la composition des phospholipides membranaires et des réserves lipidiques, une régulation des gènes liés à l'activité des transporteurs ioniques ainsi qu'un maintien de l'homéostasie métabolique. Ces modifications, également observées chez d'autres espèces d'insectes, pourraient être liées à l'augmentation de la tolérance au froid de *D. suzukii*, jouant probablement un rôle important dans sa survie hivernale et donc dans le succès de son invasion. Ces connaissances acquises sur sa thermobiologie contribueront sans doute à mieux cerner les limites physiologiques de cette espèce et prédire l'évolution de son invasion, ainsi que sa phénologie et les variations de populations au cours des saisons dans les zones déjà envahies. Mes résultats ouvrent également des perspectives intéressantes pour la mise en place de techniques de lutte intégrée contre *D. suzukii*.

Title : Underlying physiological mechanisms of thermal tolerance plasticity in the invasive fly *Drosophila suzukii*

Keywords : cold tolerance ; fluctuating temperatures ; acclimation ; omics ; pest control

Abstract : *Drosophila suzukii* is an invasive pest in Europe, North and South America. Unlike other drosophilids, females oviposit in ripe fruits that larvae consume, provoking important damages on fruit productions. The overwintering strategies of this fly are yet poorly understood. Therefore, the aim of my thesis was to acquire new fundamental knowledge about the thermal biology of this fly, and more specifically the plasticity of its thermal tolerance and the physiological mechanisms underpinning cold acclimation. In order to define its basal thermal tolerance, adults and pupae were subjected to a large set of high and low temperatures. My data confirmed that this pest was chill susceptible, and showed that survival was greatly compromised during exposures above 32°C. Next, I evaluated its thermal tolerance plasticity.

My data confirmed the high plasticity of its cold tolerance, as fluctuating thermal regimes and acclimation were able to decrease the mortality due to cold exposures.

Acclimation in this species was correlate with several physiological adjustments, such as: cryoprotectant accumulation, remodeling of membrane phospholipids and lipidic reserves, upregulation of genes linked with activity of ionic transporters and maintenance of metabolic homeostasis. Those modifications (which are shared among temperate insect species) are likely linked with cold tolerance increase provoked by acclimation. Therefore, these physiological adjustments could play an important role in its overwintering success in Europe and Canada, which can facilitate its invasion in these regions. These new data will participate to a better understanding of its physiological limits, and are thus of importance for predicting the evolution of its invasion front and its phenology and demographic variations in invaded areas. My results are also of interest regarding the set-up of integrated pest management strategies against this fly.