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Comparative Metabolic Profiling in *Drosophila suzukii* by Combined Treatment of Fumigant Phosphine and Low Temperature

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Abstract: Background/Objectives: The mechanisms of action of phosphine are diverse and include neurotoxicity, metabolic inhibition, and oxidative stress; however, its efficacy at low temperatures is unclear. **Methods**: Comparative metabolomics is suitable for investigating the response of the spotted-wing fly *Drosophila suzukii* to exposure toward a combination of cold stimuli and fumigant PH_3. **Results**: Under this combined exposure, 52 metabolites exhibiting significant differences in stress were identified and their physiological roles were analyzed in the *Drosophila* metabolic pathway. Most metabolites were involved in amino acids, TCA cycle, and nucleic acids. In addition, the alteration levels of cell membrane lipids, such as glycerophospholipids, sphingolipids, and glycerolipids, clearly showed changes in the combined treatment compared to PH_3 and low temperatures alone. Aconitic acid, a component of the TCA cycle, was completely inhibited by the combined treatment. **Conclusions**: These results suggest that treatment-specific indicators could be useful biomarkers to indicate the synergistic effects of PH_3 and low temperature on energy metabolism.

Keywords: *Drosophila suzukii*; phosphine; low temperature; metabolomics; lipidomics

1. Introduction

The spotted-wing fly *Drosophila suzukii* (Matsumara) is widely distributed in Asia, the Americas, and Europe, and is characterized by laying eggs inside fresh fruits using serrated ovipositors [1–5]. Since hatched larvae burrow into the fruit, they are difficult to detect during the early stages of infection [6–9]. Wounds caused by female ovipositing organs are entry points for secondary pathogens, such as fungi and bacteria, which further aggravate fruit damage [6–8]. This invasive behavior has made this species a serious pest worldwide, and its control is crucial at the quarantine stage [10–13].

Phosphine (PH_3) as a fumigant has been widely used to control pests in stored grains and many other stored commodities [14], but its mode of action is not well understood. PH_3, which is broken down into harmless phosphates, is very effective for controlling pests in grain storage when used as a combined treatment with carbon dioxide [15–17]. Although effective penetration into target pests, lack of residue, and low cost are the major advantages of PH_3 [18–20], long-term exposure is considered a weak point [21].

Low temperatures are primarily used to disinfect stored agricultural products from pests or for quarantine purposes [22–24]. Cold treatment has been used as a control measure against pests such as the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) [25] and Caribbean fruit fly *Anastrepha suspensa* (Loew) (Diptera:

Tephritidae) [26]. In addition, cold treatment significantly reduced adult emergence in both blueberries and strawberries, and extended the shelf life of infested fruits compared to untreated controls [27]. Therefore, low temperatures can be a good option not only for pest control, but also for maintaining the marketability of fruits [27,28].

Recent studies on fumigants have suggested the possibility of controlling pests by using combined treatments at low temperatures. Cold treatment increases the insecticidal activity of phosphine and ethyl formate against pests such as *D. suzukii* [2,5,29], the oriental fruit fly *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) [30], and the peach fruit moth *Carposina niponensis* (Lepidoptera: Carposinadae) [31]. As cold treatment is commonly performed to maintain the marketability of fruits and vegetables, combined treatment with fumigants has the advantages of time efficiency, reduced product damage, and easy control of processing conditions. Although cold fumigation has a synergistic effect on insecticidal activity, the factors that lead to this synergistic effect are not well understood. To obtain evidence of the synergistic effects in pest management, the molecular changes induced by cold conditions and PH_3 were investigated using comparative metabolic profiling.

2. Materials and Methods

2.1. Insect Rearing

The spotted-wing fly, *D. suzukii* (Matsumura) (Diptera: Drosophilidae), was provided by Dr. Bong-Su Kim (Plant Quarantine Technology Center, Animal and Plant Quarantine Agency, Gimcheon, Republic of Korea). *D. suzukii* was reared in the insect chamber at 20 ± 1 °C and $60 \pm 10\%$ relative humidity under a photo-period of 16 h light and 8 h dark [2,5,32]. The insects were maintained in a clean breeding dish (ø 100 mm × h 40 mm) supplied with artificial food and distilled water containing 20% sugar.

2.2. Phosphine and Thermal Treatment

PH_3 (Vivakill®, 2% PH_3 + 98% CO_2) was purchased from Dongbu Farm Hannong Co., Ltd. (Daejeon, Republic of Korea) and supplied by Safefume Co., Ltd. (Fumate™, 99%; Hoengseong, Republic of Korea). One hundred pupae were placed on filter paper soaked in water in a Petri dish. The experimental methods for (1) cold alone, (2) fumigation alone, and (3) combined treatments were as follows [5,32]. Cold treatment was performed at 1 °C for 24 h. PH_3 (lethal concentration time; LCT_{50}, 1.1 mg/L) was introduced at 20 °C for 4 h in a 12 L desiccator (Bibby Scientific, Staffordshire, UK) sealed with a glass stopper. The pupae were fumigated for 4 h and then immediately exposed to cold air at 1 °C for 24 h. Pupae from each group were transferred to glass vials and rapidly cooled in liquid nitro-gen to prevent metabolic changes. All treatments and controls were triplicated.

2.3. Metabolite Extraction

Whole metabolites were extracted from *D. suzukii* pupae in triplicate (100 insects/replicate). Briefly, each sample was suspended in 1 mL of the extracted solution (3:3:2, acetonitrile/isopropyl alcohol/water, *v/v/v*) and homogenized using a Taco Prep bead beater (Taco, Taichung, Taiwan) while turning it on and off at 30 s intervals for 5 min. Samples were incubated at room temperature for 20 min and centrifuged at $2500 \times g$ for 5 min at 4 °C. The supernatant was transferred to a new tube and dried under pure N_2 gas. All dried samples were suspended in 200 μL of 50% acetonitrile and sonicated for 5 min. The supernatant was filtered with 0.22 μm pore (Ultrafree-MC, Millipore, Bedford, MA, USA) and immediately loaded into the LC–QTOF/MS for metabolome analysis. The metabolite recovery rate of the sample was investigated with internal standards (L-alanine, Sigma–Aldrich, Oakville, ON, Canada), and the extraction process showed a recovery rate of 50% or greater.

2.4. Lipid Extraction

Total lipidomes were extracted from whole bodies of *D. suzukii* pupae in triplicate (100 insects/replicate) using the modified Bligh and Dyer method, as described pre-

viously [33]. Briefly, each sample was suspended in 3 mL of extracted solution (2:1, methanol/chloroform, *v/v*) and homogenized using glass beads by turning the beater on and off at 30 s intervals for 5 min. Samples were incubated at room temperature for 20 min and centrifuged at 1750× *g* for 10 min at 4 °C. Supernatants were transferred to new tubes to remove tissue debris. One milliliter of chloroform and 1.8 mL of water were added to each sample, and the mixture was vortexed for 1 min. The lower layer was separated by centrifugation at 1750× *g* for 10 min at 4 °C, followed by transferring to a new tube and drying under pure N_2 gas. Dried samples were then suspended in 200 μL of loading solution (1:1, methanol/chloroform, *v/v*) and sonicated for 5 min. Resulting supernatants were filtered with 0.22 μm pore filters and immediately loaded into the LC–QTOF/MS equipment for lipidomics. Lipid recovery rates for samples were investigated using lipid standards (SPLASH® LIPIDOMIX® Mass Spec Standard, Avanti Polar Lipids, Alabaster, AL, USA), and the extraction process showed recovery rates of 50% or greater [34].

2.5. LC-QTOF/MS

LC-QTOF/MS was performed using a liquid chromatograph triple quadrupole mass spectrometer (Agilent Technologies 1260 and 6530 System, Agilent Technologies, Santa Clara, CA, USA; Metabolomics Research Center for Functional Materials, Kyungsung University) with an electrospray ionization (ESI) source. For metabolome analysis, 5 μL of each sample was injected onto a ZORBAX Eclipse XDB-C18 column (4.6 mm × 50 mm, 1.8 μm; Agilent Technologies, Santa Clara, CA, USA) with a temperature of 55 °C. In the binary mobile phase system, phase A was water with 0.1% formic acid and phase B was acetonitrile with 0.1% formic acid. The mobile phase with a flow rate of 0.5 mL/min had the following composition conditions: initiation at 2% B, followed by a linear gradient to 2% B over 1 min, 100% B at 8 min, 100% B at 10 min, 2% B at 11 min, and 2% B at 20 min. Mass spectrometry was performed in both positive and negative modes. The capillary voltage was set to 2.0 kV in the positive mode and 1.0 kV in the negative mode. Metabolites with a mass range of *m/z* 100 to 1000 were detected using a quadrupole time-of-flight instrument.

2.6. Data Processing and Statistical Analysis

The data were analyzed in one batch to ensure that the parameters were applied equally to all samples and normalized to the total ion intensity. All entities were extracted from the LC peaks of each sample and analyzed using the Mass Hunter Qualitative soft-ware (Ver. 10.0, Agilent Technologies). All compounds were annotated using the METLIN metabolite database, filtered, scaled, and integrated using Mass Profiler Professional software (Ver. 14.0; Agilent Technologies), principal component analysis (PCA) and Pearson's correlation analysis were performed. Differentially regulated metabolites were defined as changes in compounds with values of [raw fold change (FC)] > 2 and $p < 0.01$, compared to the mock control group. Metabolites were evaluated using MetaboAnalyst 6.0 (https://www.metaboanalyst.ca) (accessed on 1 August 2024) and LIPEA (https://hyperlipea.org/home) (accessed on 1 August 2024), and relevant pathways were visualized using the Kyoto Encyclopedia of Genes and Genomes (KEGG).

3. Results and Discussion

3.1. Metabolite Changes according to Stress Conditions

Comparative metabolomics was performed to investigate the physiological effects of low temperature, PH_3, and combined treatments (low temperature and PH_3) on *D. suzukii*. When analyzing the total ion chromatogram, peaks that specifically increased or decreased compared to the control were found in each treatment group (Supplementary Figure S1A). By analysis of the mass pattern at 1.57 min, the peak was identified as L-isoleucine, which was only found in the control (Supplementary Figure S1B). A recent study found that transient isoleucine deprivation enhanced nicotine resistance and extended the lifespan of *Drosophila melanogaster* [35]. These results suggest that stresses such as low temperature and PH_3 exactly affected amino acid synthesis in the *Drosophila* metabolic

network. PCA was performed using raw FC data to investigate the reliability of the metabolic analysis (Supplementary Figure S2A). The PCA revealed an aligned cluster of metabolic data for each group and showed a significant distribution pattern in the positive (Supplementary Figure S2A-i) and negative ion modes (Supplementary Figure S2A-ii). Since the correlation analysis showed an association between each experimental group, it can be used to determine treatment-specific indicators based on altered metabolites. Low temperature, PH_3, and the combined treatment were correlated with each other and revealed the same pattern in the positive (Supplementary Figure S2B-i) and negative ion modes (Supplementary Figure S2B-ii). These results suggest that the metabolome of *D. suzukii* is clearly differentiated by low temperature, PH_3, and combined treatment.

3.2. Pathway Impact of Altered Metabolites

In total, 164 and 98 metabolites were detected in the positive and negative ion modes, respectively, and 80 indicators were filtered using an annotation process based on the metabolite database. Among them, 52 metabolites with significant differences in expression were selected for analysis of their metabolic pathways and were listed as treatment-specific indicators. Metabolites were analyzed using the enrichment ratio and pathway impact scores based on the KEGG database to examine the importance of altered metabolites in the *Drosophila* metabolic network (Figure 1). GPI-anchor and amino acid biosynthetic pathways were significantly regulated under each stress condition. In addition, metabolites related to purine and pyrimidine metabolism were modulated. The tricarboxylic acid (TCA) cycle was found to be the major metabolic network at low temperatures and PH_3 alone (Figure 1A,B), but could not be identified in the combined treatment (Figure 1C). Interestingly, fewer metabolic pathways were altered in the combined treatment than in the low temperature or PH_3. The reasons for these results are as follows: (1) only a limited number of metabolites were synergistically affected by the combined treatments; and (2) the combined treatment resulted in significant metabolic changes upon PH_3 treatment prior to cold exposure.
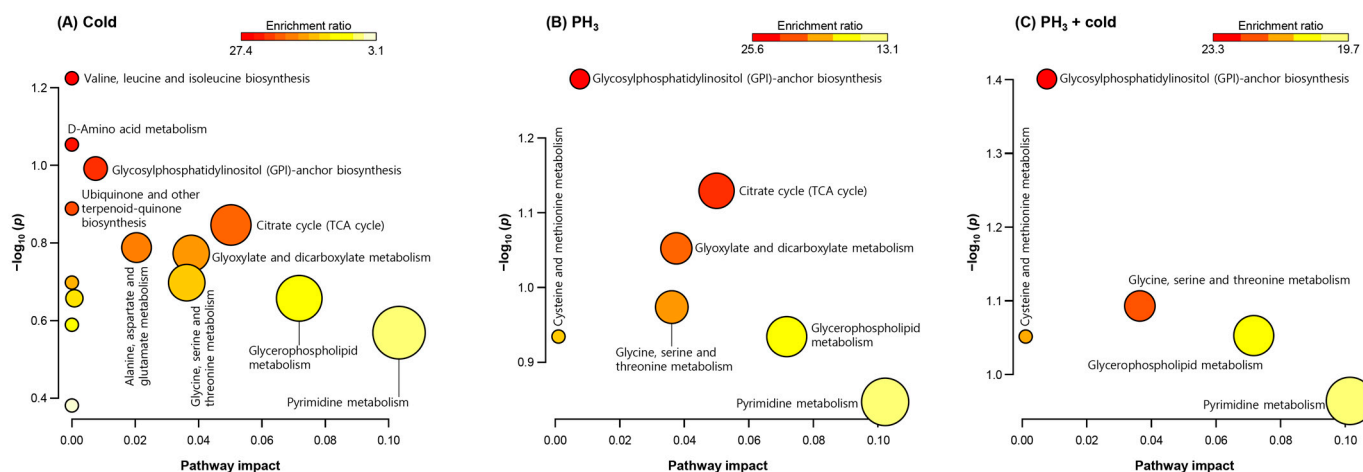

Figure 1. Enrichment ratio and pathway impact scores. Metabolite set enrichment analysis in altered metabolites. (**A**) Low temperature, (**B**) PH_3, and (**C**) combined treatment. Analysis was performed using the Kyoto Encyclopedia of Genes and Genomes database.

3.3. Treatment-Specific Metabolites as Biomarkers

Since enrichment and pathway impact scores were evaluated for the metabolites found under each stress condition, these results only showed overall tendencies. Therefore, alignment was performed to confirm which metabolites changed quantitatively in response to stress.

Metabolites detected in all treatments, but not in the mock control, were extracted as candidate treatment-specific indicators (Table 1). Metabolites involved in arachidonic acid metabolism and the immune system were identified in the PH_3 treatment. Interestingly,

3-phosphohydroxypyruvate, an intermediate between 3-phosphoglycerate and pyruvate, was detected in all stresses. 3-phosphohydroxypyruvate generates α-ketoglutarate, a major TCA cycle intermediate, during its conversion to 3-phosphoserine [36]. 3-phosphoserine generates the intermediate serine and the final product glycine. Glycine then binds to the TCA cycle intermediate succinyl-CoA. The interconversion of glutamate to α-ketoglutarate produces various amino acids, including alanine, aspartate, and arginine. Recent studies showed that low temperature and PH_3 are closely related to energy metabolism [37,38]. PH_3 induces nerve excitement by acting on acetylcholine, resulting in excessive energy consumption. Therefore, stress-inducing conditions may stimulate the production of intermediate metabolites of pyruvate, and their overproduction has a clear impact on energy metabolic pathways.

Table 1. Metabolites specifically found in each stress.

KEGG ID (PubChem CID)	Compound	Fold Change ($\log_2$)				Related Pathways
		[Mock]	[Cold]	[PH3]	[PH3 + Cold]	
C15675	Myxothiazol Z	-	5.25	-	15.82	Lipids: Polyketides
C05954	19-Hydroxy-PGB2	-	-	15.65	17.28	dme00590 Arachidonic acid metabolism
C00350	PE(18:4(6Z,9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))	-	5.09	15.62	15.81	dme00563 Glycosylphosphatidylinositol (GPI)-anchor biosynthesis dme00564 Glycerophospholipid metabolism
C03232	Phosphohydroxypyruvic acid (=3P-hydroxypyruvate)	-	18.40	18.63	18.38	dme00260 Glycine, serine, and threonine metabolism
C01092	8-Amino-7-oxononanoic acid	11.43	-	17.68	17.33	dme00780 Biotin metabolism
52924812	PE(22:4(7Z,10Z,13Z,16Z)/17:1(9Z))	5.98	-	17.91	17.59	Lipids: Glycerophospholipids
C00417	Aconitic acid (=cis-Aconitate)	19.89	19.94	6.43	-	dme00020 Citrate cycle (TCA cycle)
(614)	D-Proline	23.07	15.37	-	-	map00470 D-Amino acid metabolism
C00407	L-Isoleucine	24.81	7.50	-	-	dme00280 Isoleucine degradation dme00290 Isoleucine biosynthesis
C04778	N1-(5-Phospho-a-D-ribosyl)-5,6-dimethylbenzimidazole	15.93	10.50	-	-	dme00860 Porphyrin metabolism
C03794	N6-(1,2-dicarboxyethyl)-AMP	9.85	15.31	-	-	dme00230 Purine metabolism
(52924712)	PE(21:0/20:5(5Z,8Z,11Z,14Z,17Z))	16.66	10.06	-	-	Lipids: Glycerophospholipids
C00156	4-Hydroxybenzoic acid (=*p*-Salicylic acid)	17.06	11.37	-	-	dme00130 Ubiquinone biosynthesis
(135398700)	Xanthopterin	16.22	10.74	-	-	dme00790 Folate biosynthesis
(5312441)	13Z-Octadecenoic acid	4.61	-	-	-	dme00061 Fatty acid biosynthesis
(53480926)	LysoPE(0:0/18:2(9Z,12Z))	21.28	-	-	-	Lipids: Glycerophospholipids
(42607464)	PE(17:1(9Z)/0:0)	18.14	-	-	-	Lipids: Glycerophospholipids
C00366	Uric acid	22.22	-	-	-	dme00230 Purine metabolism

In addition, insects respond to stress by inhibiting or inactivating metabolic pathways. Therefore, the up- or downregulation of metabolites compared to the control was sorted because they can be used as indicators for each stress. The amino acids D-proline (Pro) and L-isoleucine (Ile) were detected in quantitative amounts in the mock control but not in the stressed groups. Suppression of the cryoprotectants Pro and Ile, which are known to accumulate in response to the cold in *D. melanogaster*, was contrary to previous results [39–41]. Interestingly, the downregulation of aconitic acid, an intermediate product of the TCA cycle, by low temperature and PH_3, respectively, revealed an improved inhibitory effect.

In invertebrates, PH_3 increases the signaling of the excitatory neurotransmitter acetylcholine by inhibiting acetylcholine esterase [15]. Persistent synaptic signaling by acetylcholine leads to hyperactivity, convulsions, and ultimately, excitotoxicity. PH_3 directly interferes with mitochondrial respiration and causes a lack of energy metabolism, which can be confirmed by a decrease in oxygen consumption after 4 h of exposure to PH_3 [42,43]. In addition, PH_3 acts as a reducing agent, inhibiting cytochrome c oxidase and inducing the production of hydrogen peroxide, which is a reactive oxygen species (ROS) [44,45]. These PH_3 responses ultimately resulted in metabolic inhibition, thereby supporting our finding that many metabolites were reduced or suppressed by PH_3. Collectively, these results suggest that low temperature and PH_3 share similar metabolic mechanisms that inhibit mitochondrial function and downregulate cellular metabolism.

3.4. Comparative Lipidomic Profiling by Stress

Pathway analysis revealed that the metabolites involved in glycosylphosphatidylinositol (GPI)-anchor biosynthesis were significantly regulated by low temperatures, PH_3, and combined stress (Figure 1). GPI-anchors are covalently linked to the carboxyl terminus of proteins and mediate protein attachment to lipid bilayers [46,47]. GPI, a lipid anchor for cell surface proteins, is associated with lipid rafts enriched in sphingolipids and cholesterol. Therefore, to investigate the changes in lipid profiles in response to stress, 116 lipids were identified through multivariate statistical analysis and annotation (Figure 2). PCA and correlation analyses showed that the clusters of each stress were well aligned and clearly distinguished from the mock control (Supplementary Figure S3). Most lipid classes were quantitatively altered, including fatty acids (FAs), glycerophospholipids (GPs), sphingolipids (SPs), and sterol lipids (STs), but not glycerolipids (GLs), polyketides (PKs), or prenols (PRs) (Figure 2A).

In this study, each stress condition revealed significant regulation of cell surface-related lipids, such as GPs and SPs (Table 1 and Figure 2A). A recent study has shown that lipids provide an energy source for PH_3-resistant insects to survive and an environment suitable for protecting mitochondria from PH_3 [48]. In *D. suzukii*, phospholipids in the cell membrane are mainly composed of phosphatidylethanolamine (PE) and a GP class, and low temperatures cause quantitative differences [49]. SPs, components of lipid rafts, are involved in cell membrane receptors and signal transduction, and low temperatures cause changes in the structure and profile of lipid rafts [50–52]. Low temperatures can induce changes in the phospholipid bilayer properties of cell membranes, thereby damaging their integrity [53]. These changes in membrane fluidity can lead to neuromuscular dysfunction, chills coma, and ultimately death [54–57]. Therefore, the altered levels of cell surface-related lipid GLs, GPs, and SPs are presented for each type of stress (Figure 2B). Overall, many metabolites were upregulated compared to the mock control. Heatmap analysis showed that sphingolipids were upregulated by stress and were synergistically affected by the combined treatment. Interestingly, in the GP class, PE and PS were upregulated by the combination treatment, whereas PA, PG, and PI were downregulated. Lipids are the main components of the fat body in insects and most lipids are stored in the form of triglycerides (TGs) [58,59]. In contrast, the major lipid diglyceride (DG) in insect hemolymph increases rapidly during energy requirements such as flight [59,60]. Considering the mechanism of action of PH_3 in relation to energy depletion, the increase in DG and decrease in TG in response to stress suggests that PH_3 affects the energy metabolic pathways of *D. suzukii*.
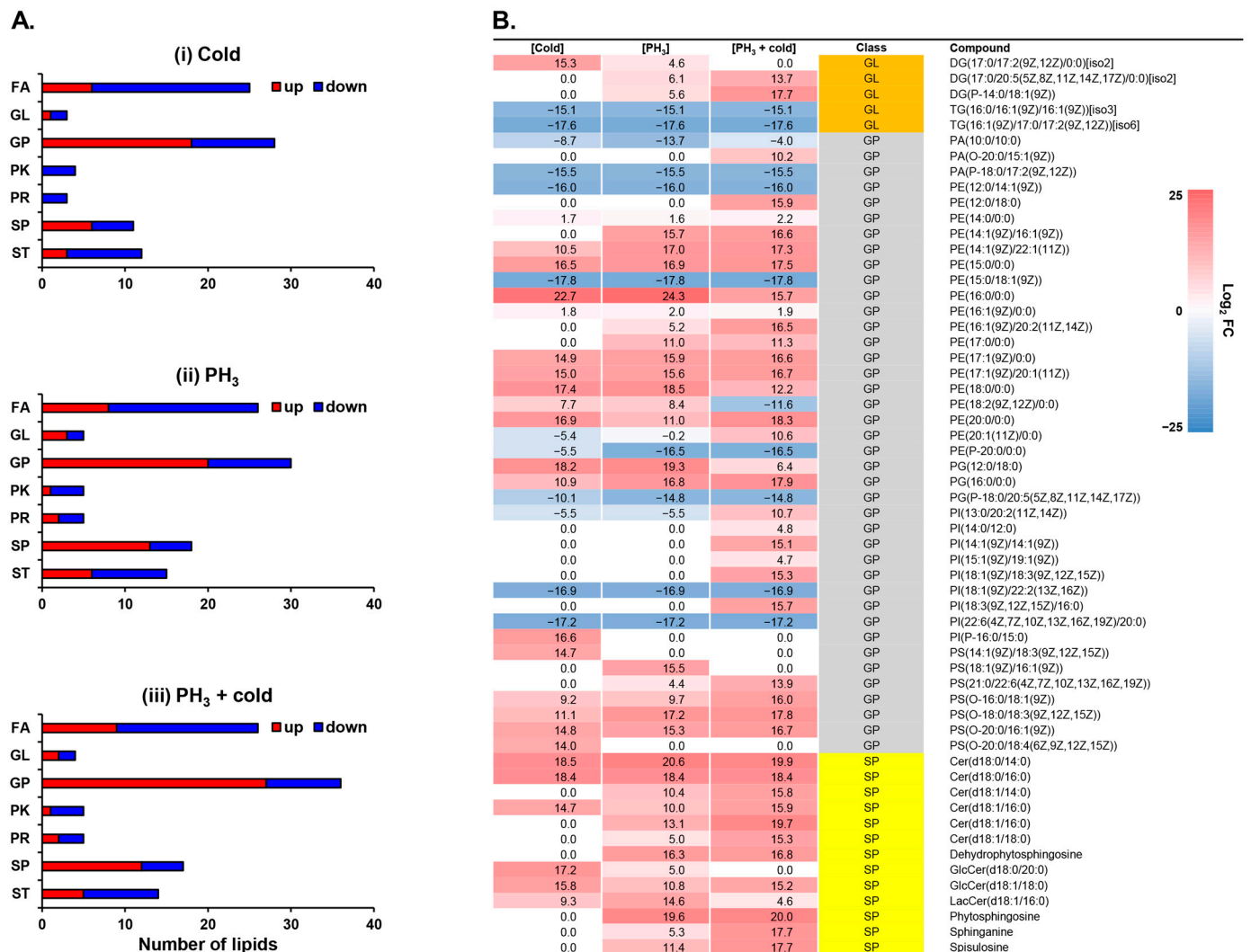
A.

B.

[Cold]	[PH₃]	[PH₃ + cold]	Class	Compound
15.3	4.6	0.0	GL	DG(17:0/17:2(9Z,12Z)/0:0)[iso2]
0.0	6.1	13.7	GL	DG(17:0/20:5(5Z,8Z,11Z,14Z,17Z)/0:0)[iso2]
0.0	5.6	17.7	GL	DG(P-14:0/18:1(9Z))
−15.1	−15.1	−15.1	GL	TG(16:0/16:1(9Z)/16:1(9Z))[iso3]
−17.6	−17.6	−17.6	GL	TG(16:1(9Z)/17:0/17:2(9Z,12Z))[iso6]
−8.7	−13.7	−4.0	GP	PA(10:0/10:0)
0.0	0.0	10.2	GP	PA(O-20:0/15:1(9Z))
−15.5	−15.5	−15.5	GP	PA(P-18:0/17:2(9Z,12Z))
−16.0	−16.0	−16.0	GP	PE(12:0/14:1(9Z))
0.0	0.0	15.9	GP	PE(12:0/18:0)
1.7	1.6	2.2	GP	PE(14:0/0:0)
0.0	15.7	16.6	GP	PE(14:1(9Z)/16:1(9Z))
10.5	17.0	17.3	GP	PE(14:1(9Z)/22:1(11Z))
16.5	16.9	17.5	GP	PE(15:0/0:0)
−17.8	−17.8	−17.8	GP	PE(15:0/18:1(9Z))
22.7	24.3	15.7	GP	PE(16:0/0:0)
1.8	2.0	1.9	GP	PE(16:1(9Z)/0:0)
0.0	5.2	16.5	GP	PE(16:1(9Z)/20:2(11Z,14Z))
0.0	11.0	11.3	GP	PE(17:0/0:0)
14.9	15.9	16.6	GP	PE(17:1(9Z)/0:0)
15.0	15.6	16.7	GP	PE(17:1(9Z)/20:1(11Z))
17.4	18.5	12.2	GP	PE(18:0/0:0)
7.7	8.4	−11.6	GP	PE(18:2(9Z,12Z)/0:0)
16.9	11.0	18.3	GP	PE(20:0/0:0)
−5.4	−0.2	10.6	GP	PE(20:1(11Z)/0:0)
−5.5	−16.5	−16.5	GP	PE(P-20:0/0:0)
18.2	19.3	6.4	GP	PG(12:0/18:0)
10.9	16.8	17.9	GP	PG(16:0/0:0)
−10.1	−14.8	−14.8	GP	PG(P-18:0/20:5(5Z,8Z,11Z,14Z,17Z))
−5.5	−5.5	10.7	GP	PI(13:0/20:2(11Z,14Z))
0.0	0.0	4.8	GP	PI(14:0/12:0)
0.0	0.0	15.1	GP	PI(14:1(9Z)/14:1(9Z))
0.0	0.0	4.7	GP	PI(15:1(9Z)/19:1(9Z))
0.0	0.0	15.3	GP	PI(18:1(9Z)/18:3(9Z,12Z,15Z))
−16.9	−16.9	−16.9	GP	PI(18:1(9Z)/22:2(13Z,16Z))
0.0	0.0	15.7	GP	PI(18:3(9Z,12Z,15Z)/16:0)
−17.2	−17.2	−17.2	GP	PI(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/20:0)
16.6	0.0	0.0	GP	PI(P-16:0/15:0)
14.7	0.0	0.0	GP	PS(14:1(9Z)/18:3(9Z,12Z,15Z))
0.0	15.5	0.0	GP	PS(18:1(9Z)/16:1(9Z))
0.0	4.4	13.9	GP	PS(21:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
9.2	9.7	16.0	GP	PS(O-16:0/18:1(9Z))
11.1	17.2	17.8	GP	PS(O-18:0/18:3(9Z,12Z,15Z))
14.8	15.3	16.7	GP	PS(O-20:0/16:1(9Z))
14.0	0.0	0.0	GP	PS(O-20:0/18:4(6Z,9Z,12Z,15Z))
18.5	20.6	19.9	SP	Cer(d18:0/14:0)
18.4	18.4	18.4	SP	Cer(d18:0/16:0)
0.0	10.4	15.8	SP	Cer(d18:1/14:0)
14.7	10.0	15.9	SP	Cer(d18:1/16:0)
0.0	13.1	19.7	SP	Cer(d18:1/16:0)
0.0	5.0	15.3	SP	Cer(d18:1/18:0)
0.0	16.3	16.8	SP	Dehydrophytosphingosine
17.2	5.0	0.0	SP	GlcCer(d18:0/20:0)
15.8	10.8	15.2	SP	GlcCer(d18:1/18:0)
9.3	14.6	4.6	SP	LacCer(d18:1/16:0)
0.0	19.6	20.0	SP	Phytosphingosine
0.0	5.3	17.7	SP	Sphinganine
0.0	11.4	17.7	SP	Spisulosine

Figure 2. Lipidomic profiling altered by exposure to low temperature and PH₃. (**A**) The number of lipids showing relative increases and decreases in *D. suzukii* after stress. (**B**) Heatmap of membrane-associated lipids. FA: fatty acid; GL: glycerolipid; GP: glycerophospholipid; PK: polyketide; PR: prenol lipid; SP: sphingolipid; ST: sterol lipid.

In addition, the metabolome set enrichment analysis revealed that sphingolipid-related metabolic pathways were primarily affected by stress (Figure 3). There was no difference between the fumigant alone and the combined treatment, but this result may be due to the effect of PH₃ already prior to the mechanism of action of low temperature on sphingolipids. These results support the reason why fewer metabolic pathways were changed in combined treatment (Figure 2).

Top 20 ranking			Pathways
[Cold]	[PH$_3$]	[PH$_3$ + cold]	
3	1	1	Sphingolipid *de novo* biosynthesis
1	3	2	Sphingolipid metabolism: integrated pathway
4	2	3	Sphingolipid metabolism
	4	4	Sphingolipid metabolism in senescence
	5	5	Neurodegeneration with brain iron accumulation (NBIA) subtypes pathway
	6	6	Synthesis of ceramides and 1-deoxyceramides
	7	7	Sphingolipid pathway
2	8	8	Phospholipid Biosynthesis
	9	9	Sphingolipid metabolism overview
	10	10	Metabolism of sphingolipids in ER and Golgi apparatus
	11	11	Starch and sucrose metabolism
5	12	12	Metabolism of lipids
6	13	13	Metabolism

Figure 3. Top 20 signaling pathways enriched at low temperature, PH$_3$, and combined treatment. Numbers and colors indicate the ranking (high: green; low: yellow) of the respective signaling pathways.

4. Conclusions

Since studies on fumigants or low temperatures in the *D. suzukii* model are individual, research on metabolic mechanisms is required to understand the synergistic effect of PH$_3$, which inhibits cytochrome oxidase activity, induces ROS production, and regulates metabolism at low temperatures. Most metabolites acted on *D. suzukii* metabolic pathways related to amino acid, lipid, and energy biosynthesis. In particular, the synergistic alteration of aconitic acid metabolites involved in the TCA cycle may be an important indicator of physiological changes in *D. suzukii*. In addition, the altered levels of the cell membrane lipids GP and SP revealed the synergistic effect of PH$_3$ and low temperatures. Since these metabolites were specifically detected in each stress condition, they can be used as indicators to determine whether the treatment was successfully performed. Therefore, this study provides useful information on treatment-specific biomarkers for low temperature or fumigation.

References

1. Atallah, J.; Teixeira, L.; Salazar, R.; Zaragoza, G.; Kopp, A. The making of a pest: The evolution of a fruit-enetrating ovipositor in *Drosophila suzukii* and related species. *Proc. R. Soc. B-Biol. Sci.* **2014**, *281*, 20132840.
2. Jeon, J.-C.; Kim, H.-K.; Koo, H.-N.; Kim, B.-S.; Yang, J.-O.; Kim, G.-H. Synergistic effect of cold treatment combined with ethyl formate fumigation against *Drosophila suzukii* (Diptera: Drosophilidae). *Insects* **2022**, *13*, 664. [CrossRef] [PubMed]
3. Karageorgi, M.; Bräcker, L.B.; Lebreton, S.; Minervino, C.; Cavey, M.; Siju, K.; Grunwald Kadow, T.C.; Gompel, C.; Prud'homme, B. Evolution of multiple sensory systems drives novel egg-laying behavior in the fruit pest *Drosophila suzukii*. *Curr. Biol.* **2017**, *27*, 847–853. [CrossRef]
4. Muto, L.; Kamimura, Y.; Tanaka, K.M.; Takahashi, A. An innovative ovipositor for niche exploitation impacts genital coevolution between sexes in a fruit-damaging *Drosophila*. *Proc. R. Soc. B-Biol. Sci.* **2018**, *285*, 20181635. [CrossRef]
5. Seok, S.-J.; Kim, H.-K.; Koo, H.-N.; Kim, G.-H. Combined Effects of Cold Treatment and Phosphine in *Drosophila suzukii* (Diptera: Drosophilidae). *Appl. Sci.* **2022**, *12*, 12531. [CrossRef]
6. Biondi, A.; Traugott, M.; Desneux, N. Special issue on *Drosophila suzukii*: From global invasion to sustainable control. *J. Pest Sci.* **2016**, *89*, 603–604. [CrossRef]
7. Goodhue, R.E.; Bolda, M.; Farnsworth, D.; Williams, J.C.; Zalom, F.G. Spotted wing drosophila infestation of California strawberries and raspberries: Economic analysis of potential revenue losses and control costs. *Pest Manag. Sci.* **2011**, *67*, 1396–1402. [CrossRef]
8. Walsh, D.B.; Bolda, M.P.; Goodhue, R.E.; Dreves, A.J.; Lee, J.; Bruck, D.J.; Walton, V.M.; O'Neal, S.D.; Zalom, F.G. *Drosophila suzukii* (Diptera: Drosophilidae): Invasive pest of ripening soft fruit expanding its geographic range and damage potential. *J. Integr. Pest Manag.* **2011**, *2*, G1–G7. [CrossRef]
9. Woltz, J.M.; Lee, J.C. Pupation behavior and larval and pupal biocontrol of *Drosophila suzukii* in the field. *Biol. Control.* **2017**, *110*, 62–69. [CrossRef]
10. Adrion, J.R.; Kousathanas, A.; Pascual, M.; Burrack, H.J.; Haddad, N.M.; Bergland, A.O.; Machado, H.; Sackton, T.B.; Schlenke, T.A.; Watada, M.; et al. *Drosophila suzukii*: The genetic footprint of a recent, worldwide invasion. *Mol. Biol. Evol.* **2014**, *31*, 3148–3163. [CrossRef]
11. Berry, J.A. Drosophila suzukii: Pathways and pathway management by regulation. In *Drosophila suzukii Management*; Springer: Cham, Switzerland, 2020; pp. 29–39.
12. Calabria, G.; Máca, J.; Bächli, G.; Serra, L.; Pascual, M. First records of the potential pest species *Drosophila suzukii* (Diptera: Drosophilidae) in Europe. *J. Appl. Entomol.* **2012**, *136*, 139–147. [CrossRef]
13. Hauser, M. A historic account of the invasion of *Drosophila suzukii* (Matsumura)(Diptera: Drosophilidae) in the continental United States, with remarks on their identification. *Pest Manag. Sci.* **2011**, *67*, 1352–1357. [CrossRef] [PubMed]
14. Alzahrani, S.M.; Ebert, P.R. Pesticidal toxicity of phosphine and its interaction with other pest control treatments. *Curr. Issues Mol. Biol.* **2023**, *45*, 2461–2473. [CrossRef] [PubMed]
15. Athié, I.; Gomes, R.A.; Bolonhezi, S.; Valentini, S.R.; De Castro, M.F.P.M. Effects of carbon dioxide and phosphine mixtures on resistant populations of stored-grain insects. *J. Stored Prod. Res.* **1998**, *34*, 27–32. [CrossRef]
16. Constantin, M.; Jagadeesan, R.; Chandra, K.; Ebert, P.; Nayak, M.K. Synergism between phosphine (PH_3) and carbon dioxide (CO_2): Implications for managing PH_3 resistance in rusty grain beetle (Laemophloeidae: Coleoptera). *J. Econ. Entomol.* **2020**, *113*, 1999–2006. [CrossRef]
17. Manivannan, S.; Koshy, G.E.; Patil, S.A. Response of phosphine-resistant mixed-age cultures of lesser grain borer, *Rhyzopertha dominica* (F.) to different phosphine-carbon dioxide mixtures. *J. Stored Prod. Res.* **2016**, *69*, 175–178. [CrossRef]
18. Nath, N.S.; Bhattacharya, I.; Tuck, A.G.; Schlipalius, D.I.; Ebert, P.R. Mechanisms of phosphine toxicity. *J. Toxicol.* **2011**, *2011*, 494168. [CrossRef]
19. Andreadis, S.S.; Athanassiou, C.G. A review of insect cold hardiness and its potential in stored product insect control. *Crop Prot.* **2017**, *91*, 93–99. [CrossRef]
20. Chaudhry, M. Review a review of the mechanisms involved in the action of phosphine as an insecticide and phosphine resistance in stored-product insects. *Pestic. Sci.* **1997**, *49*, 213–228. [CrossRef]
21. Chaudhry, M. Phosphine resistance. *Pestic. Outlook* **2000**, *11*, 88–91. [CrossRef]
22. Dohino, T.; Masaki, S.; Matsuoka, I.; Tanno, M.; Takano, T. Low temperature as an alternative to fumigation for disinfesting stored products. *Res. Bull. Plant Prot. Serv.* **1999**, *35*, 5–14.

23. Fields, P.G. The control of stored-product insects and mites with extreme temperatures. *J. Stored Prod. Res.* **1992**, *28*, 89–118. [CrossRef]

24. Mason, L.J.; Strait, C.A. Stored product integrated pest management with extreme temperatures. In *Temperature Sensitivity in Insects and Application in Integrated Pest Management*; CRC Press: Boca Raton, FL, USA, 2019; pp. 141–177.

25. De Lima, C.P.; Jessup, A.; Mansfield, E.; Daniels, D. Cold treatment of table grapes infested with Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) and Queensland fruit fly *Bactrocera tryoni* (Froggatt) Diptera: Tephritidae. *N. Z. J. Crop Hortic. Sci.* **2011**, *39*, 95–105. [CrossRef]

26. Benschoter, C. Low-temperature storage as a quarantine treatment for the Caribbean fruit fly (Diptera: Tephritidae) in Florida citrus. *J. Econ. Entomol.* **1984**, *77*, 1233–1235. [CrossRef]

27. Saeed, N.; Tonina, L.; Battisti, A.; Mori, N. Postharvest short cold temperature treatment to preserve fruit quality after *Drosophila suzukii* damage. *Int. J. Pest Manag.* **2020**, *66*, 23–30. [CrossRef]

28. El-Ramady, H.R.; Domokos-Szabolcsy, É.; Abdalla, N.A.; Taha, H.S.; Fári, M. Postharvest management of fruits and vegetables storage. *Sustain. Agric. Rev.* **2015**, *15*, 65–152.

29. Kwon, T.H.; Park, C.G.; Lee, B.-H.; Zarders, D.R.; Roh, G.H.; Kendra, P.E.; Cham, D.H. Ethyl formate fumigation and ethyl formate plus cold treatment combination as potential phytosanitary quarantine treatments of *Drosophila suzukii* in blueberries. *J. Asia-Pac. Entomol.* **2021**, *24*, 129–135. [CrossRef]

30. Burikam, I.; Sarnthoy, O.; Charernsom, K.; Kanno, T.; Homma, H. Cold temperature treatment for mangosteens infested with the oriental fruit fly (Diptera: Tephritidae). *J. Econ. Entomol.* **1992**, *85*, 2298–2301. [CrossRef]

31. Bo, L.; Fanhua, Z.; Yuejin, W. Toxicity of phosphine to *Carposina niponensis* (Lepidoptera: Carposinadae) at low temperature. *J. Econ. Entomol.* **2010**, *103*, 1988–1993. [CrossRef]

32. Lee, S.; Moon, H.H.; Kim, H.-K.; Koo, H.-N.; Kim, G.-H. Proteomic Analysis of *Drosophila suzukii* (Diptera: Drosophilidae) by Fumigant and Low-temperature Combination Treatment. *Korean J. Appl. Entomol.* **2024**, *63*, 109–117.

33. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef] [PubMed]

34. Kim, Y.-C.; Lee, J.; Lee, D.-W.; Jeong, B.-H. Large-scale lipidomic profiling identifies novel potential biomarkers for prion diseases and highlights lipid raft-related pathways. *Vet. Res.* **2021**, *52*, 1–10. [CrossRef] [PubMed]

35. Fulton, T.L.; Mirth, C.K.; Piper, M.D. Restricting a single amino acid cross-protects *Drosophila melanogaster* from nicotine poisoning through mTORC1 and GCN2 signalling. *Open Biol.* **2022**, *12*, 220319. [CrossRef]

36. Chandel, N.S. Amino acid metabolism. *Cold Spring Harbor Perspect. Biol.* **2021**, *13*, a040584. [CrossRef] [PubMed]

37. Nayak, M.K.; Daglish, G.J.; Phillips, T.W.; Ebert, P.R. Resistance to the fumigant phosphine and its management in insect pests of stored products: A global perspective. *Annu. Rev. Entomol.* **2020**, *65*, 333–350. [CrossRef]

38. Storey, K.B.; Storey, J.M. Insect cold hardiness: Metabolic, gene, and protein adaptation. *Can. J. Zool.* **2012**, *90*, 456–475. [CrossRef]

39. Colinet, H.; Larvor, V.; Laparie, M.; Renault, D. Exploring the plastic response to cold acclimation through metabolomics. *Funct. Ecol.* **2012**, *26*, 711–722. [CrossRef]

40. Koštál, V.; Korbelová, J.; Rozsypal, J.; Zahradníčková, H.; Cimlová, J.; Tomčala, A.; Šimek, P. Long-term cold acclimation extends survival time at 0°C and modifies the metabolomic profiles of the larvae of the fruit fly *Drosophila melanogaster*. *PLoS ONE* **2011**, *6*, e25025. [CrossRef] [PubMed]

41. Koštál, V.; Zahradníčková, H.; Šimek, P. Hyperprolinemic larvae of the drosophilid fly, *Chymomyza costata*, survive cryopreservation in liquid nitrogen. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 13041–13046. [CrossRef]

42. Chefurka, W.; Kashi, K.; Bond, E. The effect of phosphine on electron transport in mitochondria. *Pest. Biochem. Physiol.* **1976**, *6*, 65–84. [CrossRef]

43. Price, N. The effect of phosphine on respiration and mitochondrial oxidation in susceptible and resistant strains of *Rhyzopertha dominica*. *Insect Biochem.* **1980**, *10*, 65–71. [CrossRef]

44. Bolter, C.J.; Chefurka, W. Extramitochondrial release of hydrogen peroxide from insect and mouse liver mitochondria using the respiratory inhibitors phosphine, myxothiazol, and antimycin and spectral analysis of inhibited cytochromes. *Arch. Biochem. Biophys.* **1990**, *278*, 65–72. [CrossRef] [PubMed]

45. Schlipalius, D.I.; Valmas, N.; Tuck, A.G.; Jagadeesan, R.; Ma, L.; Kaur, R.; Goldinger, A.; Anderson, C.; Kuang, J.; Zuryn, S. A core metabolic enzyme mediates resistance to phosphine gas. *Science* **2012**, *338*, 807–810. [CrossRef]

46. Borges, A.R.; Link, F.; Engstler, M.; Jones, N.G. The glycosylphosphatidylinositol anchor: A linchpin for cell surface versatility of trypanosomatids. *Front. Cell. Dev. Biol.* **2021**, *9*, 720536. [CrossRef]

47. Kinoshita, T. Glycosylphosphatidylinositol (GPI) anchors: Biochemistry and cell biology: Introduction to a thematic review series. *J. Lipid Res.* **2016**, *57*, 4–5. [CrossRef]

48. Alnajim, I.; Aldosary, N.; Agarwal, M.; Liu, T.; Du, X.; Ren, Y. Role of lipids in phosphine resistant stored-grain insect pests *Tribolium castaneum* and *Rhyzopertha dominica*. *Insects* **2022**, *13*, 798. [CrossRef] [PubMed]

49. Enriquez, T.; Colinet, H. Cold acclimation triggers lipidomic and metabolic adjustments in the spotted wing drosophila *Drosophila suzukii* (Matsumara). *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **2019**, *316*, R751–R763. [CrossRef]

50. Bieberich, E. Sphingolipids and lipid rafts: Novel concepts and methods of analysis. *Chem. Phys. Lipids.* **2018**, *216*, 114–131. [CrossRef] [PubMed]

51. Rajendran, L.; Simons, K. Lipid rafts and membrane dynamics. *J. Cell Sci.* **2005**, *118*, 1099–1102. [CrossRef]

52. van Meer, G.; Lisman, Q. Sphingolipid transport: Rafts and translocators. *J. Biol. Chem.* **2002**, *277*, 25855–25858. [CrossRef]
53. Koštál, V. Cell structural modifications in insects at low temperature. In *Low Temperature Biology of Insects*; Cambridge University Press: Cambridge, MA, USA, 2010; pp. 116–140.
54. Hosler, J.S.; Burns, J.E.; Esch, H.E. Flight muscle resting potential and species-specific differences in chill-coma. *J. Insect Physiol.* **2000**, *46*, 621–627. [CrossRef] [PubMed]
55. Lee, R.E. A primer on insect cold-tolerance. In *Low Temperature Biology of Insects*; Cambridge University Press: Cambridge, MA, USA, 2010; pp. 3–34.
56. MacMillan, H.A.; Sinclair, B.J. Mechanisms underlying insect chill-coma. *J. Insect Physiol.* **2011**, *57*, 12–20. [CrossRef] [PubMed]
57. Yocum, G.D.; Žďárek, J.; Joplin, K.H.; Lee, R.E., Jr.; Smith, D.C.; Manter, K.D.; Denlinger, D.L. Alteration of the eclosion rhythm and eclosion behavior in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress. *J. Insect Physiol.* **1994**, *40*, 13–21. [CrossRef]
58. Ad, M.; Van der Horst, D.J.; Van Marrewijk, W.J. Insect lipids and lipoproteins, and their role in physiological processes. *Prog. Lipid Res.* **1985**, *24*, 19–67.
59. Arrese, E.L.; Soulages, J.L. Insect fat body: Energy, metabolism, and regulation. *Annu. Rev. Entomol.* **2010**, *55*, 207–225. [CrossRef]
60. Jutsum, A.; Goldsworthy, G. Fuels for flight in Locusta. *J. Insect Physiol.* **1976**, *22*, 243–249. [CrossRef]