Renal neuroendocrine control of desiccation and cold tolerance by Drosophila suzukii

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Abstract

BACKGROUND: Neuropeptides are central to the regulation of physiological and behavioural processes in insects, directly impacting cold and desiccation survival. However, little is known about the control mechanisms governing these responses in Drosophila suzukii. The close phylogenetic relationship of D. suzukii with Drosophila melanogaster allows, through genomic and functional studies, an insight into the mechanisms directing stress tolerance in D. suzukii.

RESULTS: Capability (Capa), leucokinin (LK), diuretic hormone 44 (DH44) and DH31 neuropeptides demonstrated a high level of conservation between D. suzukii and D. melanogaster with respect to peptide sequences, neuronal expression, receptor localisation, and diuretic function in the Malpighian tubules. Despite D. suzukii’s ability to populate cold environments, it proved sensitive to both cold and desiccation. Furthermore, in D. suzukii, Capa acts as a desiccation- and cold stress-responsive gene, while DH44 gene expression is increased only after desiccation exposure, and the LK gene after nonlethal cold stress recovery.

CONCLUSION: This study provides a comparative investigation into stress tolerance mediation by neuroendocrine signalling in two Drosophila species, providing evidence that similar signalling pathways control fluid secretion in the Malpighian tubules. Identifying processes governing specific environmental stresses affecting D. suzukii could lead to the development of targeted integrated management strategies to control insect pest populations.

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Keywords: Drosophila; neuropeptides; Malpighian tubules; desiccation; cold tolerance

1 INTRODUCTION

Drosophila suzukii (Matsumura), also known as the spotted wing Drosophila, is a serious pest of soft fruit worldwide.1 Although originally of Asian origin, the species has since become invasive in North America and continental Europe.2–4 Drosophila suzukii females inflict substantial crop damage by ovipositing eggs into ripe, pre-harvest fruits via a specialised serrated ovipositor.5,6 This damage is then exacerbated by the hatched larvae feeding on the fruit, resulting in substantial economic losses.7 Compared with D. melanogaster, the physiological responses of Drosophila melanogaster to environmental stressors such as cold and desiccation have been well characterised.8 Drosophila melanogaster, which phylogenetically is very closely related to D. suzukii, is known to be chill susceptible and incapable of surviving internal ice formation.9 However, adult females can ‘overwinter’, that is, survive periods of sustained cold temperatures and associated desiccation, by entering a weak diapause, that is intermediate between a true photoperiodic diapause and a temperature-driven quiescence, whereby they depress their metabolic rate, accumulate energy reserves, suppress ovarian development, and increase melanisation of the cuticle.10,11 To survive the multiple-stress environment of winter, Drosophila species exhibit enhanced stress tolerances and have many physiological and genetic mechanisms underlying cold and desiccation tolerance. Both cold and desiccation are closely linked at the molecular level to adjustments in the expression of genes involved in key physiological responses, for example in ion transport, carbohydrate metabolism, antioxidant production, immunity, signalling and gene expression pathways.12–17 Recent work has identified neuroendocrine signalling as a control mechanism of environmental stress responses.18–21

The ability to rapidly respond to, and tolerate over time, abiotic stresses such as temperature fluctuations (and associated alterations in food/water availability), in the short term and/or as a seasonal variation, requires insect osmoregulatory systems, including the gut and Malpighian (renal) tubule (MT), to act in a dynamic and appropriate manner to effect the physiological processes necessary to ensure the animals’ survival. Insect osmoregulation, that is, the regulation of cellular ion and water homeostasis via transporters and water channels, is subject to highly sophisticated endocrine control.22 In D. melanogaster, several families of neuropeptides have been identified that regulate diuresis in the MTs, including the diuretic hormones DH31,23 and DH44,24 the Capability (Capa)-related peptides25 and the

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leukokinin (LK) neuropeptide.26 The recent transcriptomics and peptidomic analyses of the neuropeptides and their cognate receptors in D. suzukii have allowed identification of orthologous peptides and receptors, which in turn facilitates functional studies to identify suitable targets for the development of novel control strategies against this invasive pest.27

The depth of knowledge garnered from the genetic model organism D. melanogaster can readily be used to identify fundamental similarities that may exist in D. suzukii. Here, we describe the peptide sequences, morphology and organisation of the Capa, LK, and DH44-producing neurons in the larval and adult central nervous system (CNS) of D. suzukii to gain better insight into the structure–function relationship of these neuropeptides. We also determined the MT-specific cell types that receive Capa, LK, DH44 and DH31 neuropeptide signals and their physiological role in modulating tubule fluid homeostasis. Furthermore, we investigated tolerance to desiccation and cold in both D. melanogaster and D. suzukii, and we provide data suggesting that neuroendocrine responses to these stresses are potentially altered by the action of neuropeptides acting on the MT to regulate water and ion homeostasis critical for the animal’s survival.

2 MATERIALS AND METHODS

2.1 Fly stocks

Drosophila melanogaster wild-type Canton-S (CS) flies were obtained from Bloomington Stock Center (Indiana University, Bloomington, USA) and maintained on a standard Drosophila medium at 22 °C (unless otherwise stated) and 45-55% relative humidity with a 12:12 h light:dark photoperiod. Drosophila suzukii flies, originally collected in Italy, were obtained from the laboratory of R. Elwyn Isaac (University of Leeds, Leeds, UK) and reared on standard cornmeal agar medium supplemented with a blue-berry fruit, at 26 °C and 60% humidity with a 12:12 h light:dark photoperiod. Adult Drosophila (1-2 weeks post-eclosion) of both sexes were used in this study.

2.2 Immunostaining

Immunostaining procedures were performed as described previously.25 Adult MTs and larval and adult nervous systems were dissected in Schneider’s medium (Thermo Fisher Scientific, Paisley, UK) and fixed with 4% (w/v) paraformaldehyde (PAF) for 30 min at room temperature. Rhodamine-labelled purified rabbit anti-DH44 (1:200024), rhodamine-labelled purified rabbit anti-Capa precursor peptide (dilution 1:50025), and rabbit anti-LK (1:100026) were used. Alexa Fluor 405/564-conjugated affinity-purified goat anti-mouse and anti-rabbit antibodies (Thermo Fisher Scientific) were used in a dilution of 1:1000 for visualisation of the primary antisem. Incubations in the primary and secondary antibodies were performed overnight. Tubules were incubated with the nuclear stain 4’,6-diamidino-2-phenylindole (DAPI; 1

2.3 Peptide synthesis and fluorescent-tagged neuropeptide labelling

Drosophila Capa-1, LK, DH44 and DH31 (both with and without N-terminal cysteine) were synthesised by Cambridge Peptides (Birmingham, UK). The amino acid sequences used to synthesise the neuropeptides were based on the D. melanogaster sequences as most of the peptides predicted and identified from D. suzukii appear to be identical to those previously characterised from D. melanogaster.27 The protocol and generation of neuropeptide conjugated to a high quantum yield fluorophore have been described previously.28 The fluorescent-tagged neuropeptides generated were TMR-C5-maleimide-Capa-1, Alexa-488-C5-maleimide-LK, TMR-C5-maleimide-DH44 and TMR-C5-maleimide-DH31. Ligand receptor binding assays were performed on live MTs dissected from 6 – 7-day-old adult females. Tubules were mounted on poly-L-lysine coated glass-bottomed dishes in phosphate-buffered saline (PBS) and then incubated for 2 minutes in a 1:1 mixture of Schneider’s medium:Drosophila saline containing 500 ng/ml DAPI and 10-7 M fluorescent-tagged neuropeptide and subsequently with 10-3 M unlabelled neuropeptides. As the staining is transitory, the samples were imaged immediately after treatment. All images were taken using a Zeiss LSM 810 inverted confocal microscope.

2.4 Fluid secretion assay

Secretion assays were performed as described previously.29 MTs from 6 – 7-day-old adult female D. suzukii flies were dissected under Schneider’s medium and isolated into 10-μl drops of a 1:1 mixture of Schneider’s medium:Drosophila saline. Intact tubules were left to secrete for approximately 30 min, with non-secreting tubules being replaced if necessary, to produce a set of 10 – 15 working tubules. Secretion rates were measured every 10 min, then the diuretic Drosophila Capa-1, LK, DH44 and DH31, neuropeptides were added to 10-3 M, and secretion rates were measured for a further 30 min. Data are plotted as mean ± standard error of the mean (SEM).

2.5 Gravimetric method of estimating body water

To measure wet body weight, D. melanogaster and D. suzukii 6-day-old normally fed female flies were anaesthetised and weighed on an AND GR-202 (Oxford, UK) precision balance (analytical weighing to within 0.0001 g). Another group of flies of each Drosophila species were weighed after 18 h of desiccation with no food and no water. Water loss over 18 h was calculated for each group by subtracting the water content at 18h from that at 0h. Experiments were run in triplicate with at least 10 flies of each Drosophila species.

2.6 Assays of gene expression for cold and desiccation stress

2.6.1 Cold

Drosophila suzukii 6-day-old female flies were untreated (control), chilled at 4 °C for 6 h (cold), or chilled at 4 °C for 6 h and subsequently allowed to recover for 24 h (recovery). Before treatment, both D. suzukii and D. melanogaster were maintained at a culture temperature of 26 °C to enable a direct species comparison and avoid any potential effects of rearing temperature on inherent thermal tolerance. No mortality was observed for the different treatment groups.

2.6.2 Desiccation

Drosophila suzukii 6-day-old female flies were either untreated (control), placed in empty vials (no food or water) for 18 h at 21 °C and 45-55% relative humidity (desiccation), or exposed to
desiccation for 18 h and subsequently allowed to recover for 24 h (recovery). Before treatment, both *D. suzukii* and *D. melanogaster* were maintained at a culture temperature of 26 °C to enable a direct species comparison and avoid any potential effects of rearing temperature and relative humidity on inherent thermal and desiccation resistance. Following treatment, groups of 10 flies were transferred immediately to microcentrifuge tubes containing TRIzol Reagent (Thermo Fisher Scientific) for RNA extraction.

For *D. suzukii* whole-fly mRNA expression, quantitative reverse transcriptase – polymerase chain reaction (qRT-PCR) amplifications were performed in an ABI StepOnePlus Detection System (Applied Biosystems, CA, USA) with Brilliant III Ultra-Fast SYBR Green QPCR master mix (Agilent, Edinburgh, UK) using the following primer pairs: *Capa*, 5′-CCGAGTCTGGCAACAGTCTG-3′ and 5′-GACACAAACAGGACAAAGG-3′; *LK*, 5′-CAGTTCTGGACAGGATCGGAG-3′ and 5′-CAGGTCTCGCATTGTAGGCATC-3′; and 5′-ATGCCAAGCCAATCCAATGGTG-3′ and 5′-TGCGTCCAAAGCGAGTTGC-3′.

Sequences were obtained from DINeR (Applied Biosystems, CA, USA) with Brilliant III Ultra-Fast SYBR Green QPCR master mix (Agilent, Edinburgh, UK) using the following primer pairs:

- 5′-GTTGCTCCAAACCCTCCA-3′ and 5′-GATCCGTCTAACACCTCCC-3′.
- 5′-CCGAGTCTGGCAAACAGTCTG-3′ and 5′-GGTTGCTCCAAACCCTCCA-3′.
- 5′-ATGCCAAGCCAATCCAATGGTG-3′ and 5′-TGCGTCCAAAGCGAGTTGC-3′.

Ribosomal protein L18 (rpl18), 5′-TTGCTCCAAACCCTCCA-3′ and 5′-GATCGGCTCAACACCTCCC-3′: Data were normalised against the *D. suzukii* rpl18 standard and expressed as fold change compared with controls ± SEM (n = 3).

### 2.7 Survival under different stressors

#### 2.7.1 Cold

Cold tolerance was determined via calculation of the lower lethal temperature (LLT50), i.e. the low temperature that results in 50% mortality of a test population. Adults of *D. suzukii* and *D. melanogaster*, both reared at 26 °C, were selected at 6 days post-eclosion, respectively, and exposed to a range of low temperatures (-10°C to -2°C at 1 °C intervals) using a direct plunge method.30,31 Temperature ranges were selected to encompass 0 – 100% mortality. For each temperature treatment, 30 adults of each sex and each species were anesthetised briefly with CO2 and placed in Eppendorf tubes at densities of ten adults per tube, which, in turn, were placed within a glass boiling tube held within an alcohol bath (Haake G50 and PC200; Thermo Scientific, Karlsruhe, Germany) pre-set to the desired temperature. Adults were held at the desired exposure temperature for 1 h. Following exposure, adults were allowed to recover in culture vials containing a food source and survival was assessed after 48 h. The procedure was repeated for each exposure temperature.

#### 2.7.2 Desiccation

*Drosophila suzukii* and *D. melanogaster* 6-day-old male and female flies were anesthetised briefly with CO2 and placed in Eppendorf tubes at densities of ten adults per tube, which, in turn, were placed within a glass boiling tube held within an alcohol bath (Haake G50 and PC200; Thermo Scientific, Karlsruhe, Germany) pre-set to the desired temperature. Adults were held at the desired exposure temperature for 1 h. Following exposure, adults were allowed to recover in culture vials containing a food source and survival was assessed after 48 h. All experiments were run in triplicate with at least 30 flies in each run for each *Drosophila* species.

### 2.8 Bioinformatics

Neuropeptide precursor sequences were obtained from DinEr (http://www.neurotresspep.eu/diner/) and/or from the National Center for Biotechnology Information (NCBI). Sequences were aligned using the multiple sequence alignment program Clustal Omega.33

### 2.9 Statistical analysis

For fluid secretion analysis and for the water retention assay, a two-tailed Student’s t-test, taking *P* = 0.05 as the critical value (for two independent groups: basal versus stimulated), was used. For mRNA level quantification, one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons of means with a significance level of *P* < 0.05 (for three independent groups: non-stressed versus stressed versus recovered) was used to compare neuropeptide mRNA levels in cold or desiccated flies. For survival curves obtained in desiccation assays, significance was assessed by the log-rank (Mantel–Cox) test. Log-rank tests were conducted for each pairwise comparison. The statistical tests were performed using GraphPad Prism 7.0 software (GraphPad Software Inc., CA, USA). For cold shock experiments, the temperature resulting in 50% mortality of experimental populations (LLT50) was determined for low exposure temperatures using Probit analysis in MINITAB, version 17 (Minitab Inc., State College, PA, USA). Handling controls resulted in approximately 99% survival across all treatments. The natural response rate was therefore assumed to be close to zero and was not included in the model. Significant differences in mortality were identified by non-overlapping 95% fiducial limits.

### 3 RESULTS

#### 3.1 Neuropeptide precursor data

Amino acid BLAST searches of the *D. suzukii* protein databases revealed the precursor sequences of *Capa*,34 *LK*, *DH44* and *DH31* neuropeptide families. A comparison of *D. suzukii* and *D. melanogaster* *Capa*, *LK*, *DH44* and *DH31* peptide precursors is shown in Fig. 1. We found that the amino acid sequences of the *D. suzukii* predicted peptides are identical to the sequences found in *D. melanogaster*. Furthermore, the presence of these peptides and/or peptides precursors in *D. suzukii* has previously been confirmed by mass spectrometric analysis.27

#### 3.2 *Capa*, *LK* and *DH44* neuropeptide-expressing neurons

To gain insight into the physiological function of *Capa*, *LK* and *DH44* neuropeptides in *D. suzukii*, we determined the localisation of the neurons producing these neuropeptides and their projections. As the peptide precursor sequences are very similar between *D. suzukii* and *D. melanogaster*, we used available antisera against *D. melanogaster* *Capa*, *LK*, and *DH44* on the *D. suzukii* larval and adult CNS.

The antiserum to the *Capa* precursor recognised several neurons in the larval and adult CNS (Fig. 2a and b), consisting of three pairs of ventral neuroendocrine cells in the abdominal neuromeres (Va neurons), and a single pair of large neuroendocrine cells in the suboesophageal ganglion (SEG neurons). In the larva, the Va neurons use the surface of the three pairs of abdominal median nerves as their neurohaemal release site (Fig. 2a, arrowhead), while in the adult, the dorsal surface of the thoracoabdominal ganglion is used. In larvae and adults, the axons of the SEG neurons project to the cerebral ganglion and from the brain by means of the nervi corporis cardiac to the retrocerebral complex (Fig. 2a, arrowhead), which allows the release of the *Capa* neuropeptides into the haemolymph.

In this study, *LK*-immunoreactive cells were identified using an antibody prepared against leucokinin I, which shows broad specificity for the *LK* family of neuropeptides.26,35 *LK* expression was observed in the brain and ventral ganglion of the *D. suzukii* CNS (Fig. 2c and d). One pair of large protocerebrum neurons located in the lateral horn leucokinin (LHLLK) area surrounds the peduncles of the mushroom bodies and two pairs of suboesophageal leucokinin (SELK) neurons project extended processes to the tritocerebrum and through the cervical connection to the ventral
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Figure 1. Alignment of amino acid sequences encoded by D. suzukii (Drosu) and D. melanogaster (Drome) neuropeptide precursors identified from NCBI for (a) Capa, (b) leucokinin (LK), (c) DH44, and (d) DH31 prepropeptides. Predicted peptide sequences are highlighted in bold, potential endoproteolytic cleavage sites are in italics, and the signal peptide sequence is underlined. GenBank Accession numbers: Drome-Capa (AAF56969.2), Drosu-Capa (XP_016937441.1), Drome-LK (AAF497331.2), Drosu-LK (XP_016933307.1), Drome-DH44 (NP_649922.2), Drosu-DH44 (XP_016930975.1), Drome-DH31 (Q9VLK4.1) and Drosu-DH31 (XP_016944990.1).

ganglion. In the larva, the ventral ganglion showed one pair of prominent LK-immunoreactive neurons in seven abdominal leucokinin (ABLK) neurones that send their processes to the lateral abdominal nerves (Fig. 2c, arrowhead). In the ventral ganglion of the adult, around nine pairs of abdominal neurones have axons that project from the CNS via the abdominal nerves and processes that connect to each other, both ipsi- and contra-laterally. The latter neurones appear to be primarily responsible for the release of LK into the haemolymph.

Using a specific anti-DH44 antibody, DH44 expression was observed in a restricted number of neurones within the larval and adult CNS (Fig. 2e and f), most notably in a bilateral triplet of...
Figure 2. Immunocytochemical localisation of Capa, LK and DH44 neuropeptides in the *D. suzukii* larval and adult central nervous system (CNS). (a, b) Localisation of Capa neurons using anti-Capa precursor antibody in (a) the larval and (b) the adult CNS. Capa precursor immunoreactivity is localised to a single pair of very large neuroendocrine cells in the suboesophageal ganglion (SEG neurons) and three pairs of abdominal neuroendocrine cells (Va neurons). Arrowheads show neurohaemal organs, the retrocerebral complex for the two cell bodies in the suboesophageal neuromere, and the abdominal median nerves for the three pairs of ventral neuroendocrine cells in the abdominal neuromeres. (c, d) LK immunofluorescence using anti-LK antibody in (c) the larval and (d) the adult CNS. LK-expressing neurons are distributed as a pair of lateral horn LK (LHLK) neurons, two pairs of suboesophageal LK (SELK) neurons, and seven pairs in the abdominal ganglia of the larval CNS. In the ventral ganglion of the adult, around nine pairs of abdominal LK (ABLK) neurons have axons that leave the CNS by abdominal nerves and processes for the release of LK into the haemolymph. (e, f) Localisation of DH44 neurons using anti-DH44 antibody in (e) the larval and (f) the adult CNS. DH44 peptides are produced in six neurons (three cells in each cerebral lobe) of the pars intercerebralis (PI), with axons running to the retrocerebral complex of the corpus cardiacum (arrow). Additional smaller set of neuroendocrine cells in the larval and adult ventral nerve cord are labelled with the DH44 antibody. All patterns of expression are representative of both males and females. Inserts are maximum projections of confocal z-series showing magnification of selected regions. VNC, ventral nerve cord; TAG, thoracic-abdominal ganglion. Scale bars = 100 μm.
circulation from neurohaemal release sites, whereupon they bind. As in DH44 and DH31 neuropeptides specific to the PCs, with LK confined (Fig. 3). These data are consistent with the known action of Capa-1, DH44 and DH31 neuropeptides to tubule PCs and SCs. As in D. melanogaster, application of Capa-1-F, DH44-F and DH31-F maps their receptors to the basolateral membrane of PCs (Fig. 3a, c and d), whereas LK-F specifically labels SCs (Fig. 3b). Specificity of the fluorescently tagged neuropeptide binding was verified by a ligand competition assay in which the respective unlabelled peptide was able to almost fully abolish the fluorescent signal by a ligand competition assay in which the respective unlabelled peptide was able to almost fully abolish the fluorescent signal (Fig. 3a). These data are consistent with the known action of Capa-1, DH44 and DH31 neuropeptides specific to the PCs, with LK confined to SCs, in Drosophila MTs where they act through independent, although complimentary, cell signalling pathways to increase primary urine production.37

3.3 Receptor mapping assay using fluorescently labelled neuropeptides
It has previously been demonstrated in D. melanogaster that Capa, LK, DH44 and DH31, neuropeptides are released into the circulation from neurohaemal release sites, whereupon they bind their cognate G protein-coupled receptors localised at the MTs.35 The MTs are characterised by two main physiologically distinct secretory cell types, the principal (PC) and secondary (stellate; SC) cells, which receive multiple separate neuropeptide signals.28 Employing a fluorescent ligand-receptor binding assay to determine the neuropeptide signalling associated with these two cell types in D. suzukii MTs, we were able to demonstrate specificity of binding of Capa-1 (one of the peptides encoded by the Capa gene), LK, DH44 and DH31 neuropeptides to tubule PCs and SCs. As in D. melanogaster, application of Capa-1-F, DH44-F and DH31-F maps their receptors to the basolateral membrane of PCs (Fig. 3a, c and d), whereas LK-F specifically labels SCs (Fig. 3b). Specificity of the fluorescently tagged neuropeptide binding was verified by a ligand competition assay in which the respective unlabelled peptide was able to almost fully abolish the fluorescent signal (Fig. 3). These data are consistent with the known action of Capa-1, DH44 and DH31 neuropeptides specific to the PCs, with LK confined to SCs, in Drosophila MTs where they act through independent, although complimentary, cell signalling pathways to increase primary urine production.37

3.4 Effect of Capa, LK, DH44 and DH31, neuropeptides on fluid secretion
Drosophila melanogaster Capa-1, LK, DH44 and DH31, neuropeptides were tested on their ability to stimulate fluid secretion on D. suzukii MTs. As expected, all four neuropeptides significantly increased the fluid secretion rate over 30 min (Fig. 4 a – d), with the extent of these increases comparable to those observed in D. melanogaster tubules. Interestingly, the greatest stimulation of fluid secretion was induced by LK, a neuropeptide that modulates the ‘chloride shunt’ pathway specifically in D. melanogaster SCs, resulting in a rapid collapse of the transepithelial potential and a concomitant increase in fluid secretion.38,39 DH44, DH31, and the Capa-1 neuropeptides act on their cognate G protein coupled receptor localised in the PCs. Activation of the PCs alone produces only a modest increase in fluid secretion, as the resting chloride conductance remains relatively low. The impact of desiccation on MT function was also assessed via secretion assays. The baseline and neuropeptide-stimulated secretion rate of D. suzukii, exposed to 18 h of desiccation, was not significantly lower than that of unstressed flies. Indeed, the percentage change in secretion rates following stimulation with the different neuropeptides was similar in desiccated and non-desiccated flies (Fig. 4). Intriguingly, while neuropeptides significantly increase the fluid secretion rate to a similar extent when applied to MTs of the two species exposed to desiccation,21,28 the baseline secretion rates are significantly lower in D. melanogaster flies; this suppression of basal fluid secretion is not apparent in D. suzukii. Hence, the direct action of these neuropeptides to D. suzukii MTs suggests that excretory water loss may contribute significantly to desiccation resistance.

3.5 Desiccation and cold tolerance
As Capa and kinin have been previously shown to modulate stress tolerance in D. melanogaster, we investigated comparative survival following desiccation and cold stress between D. melanogaster and D. suzukii male and female flies. During desiccation stress, D. melanogaster survived longer than D. suzukii, with a significant sexually dimorphic effect observed for both Drosophila species, with females exhibiting enhanced desiccation resistance (Fig. 5a; median survival time: D. melanogaster males, 39 h; D. melanogaster females, 25 h; D. suzukii males, 17 h; D. suzukii females, 11 h). In desiccating environments, a key mechanism used by insects to maintain water balance is to reduce the rate of water loss.40 We then performed water loss measurements in flies desiccated for 18 h compared with normally fed female flies for both Drosophila species (Fig. 5b; body weight of individual adult female
fly (mean ± SEM): D. melanogaster, 1.02 ± 0.05 mg; D. melanogaster desiccated, 0.86 ± 0.04 mg; D. suzukii, 1.89 ± 0.16 mg; D. suzukii desiccated, 1.51 ± 0.13 mg). During desiccation stress, water loss in D. suzukii was significantly higher than in D. melanogaster (20% and 15%, respectively, compared with nonstressed control groups; P < 0.05). During cold exposure, D. melanogaster proved significantly more cold tolerant than D. suzukii, as determined by non-overlapping 95% fiducial limits (Fig. 5c). No significant difference between the cold tolerance of males and that of females was observed for either species (LLT50: D. melanogaster females, -6.1 °C; D. melanogaster males, -5.9 °C; D. suzukii females, -5.5 °C; D. suzukii males, -5.2 °C). For D. melanogaster, 5 – 95% mortality occurred over a temperature range of 1.1 °C for females and 1.9 °C for males. For D. suzukii, the corresponding mortality level occurred over a temperature range of 3.7 and 4.8 °C, respectively.

3.6 Effect of desiccation and cold stress on neuropeptide mRNA levels
MTs are critical organs for epithelial fluid transport and stress tolerance in insects, and recent studies have described the mechanism in renal tubule epithelia that enhances both desiccation and cold survival.20,61 Given that tubule function is controlled by multiple neuropeptides, putative roles for Capa, LK, DH44, and DH31, signalling in desiccation and cold stress were explored (Fig. 6) by measuring their mRNA expression levels, after exposure to 18 h of desiccation at 21 °C or after exposure to a cold shock of 4 °C for 6 h, or after exposure to desiccation/cold stress and subsequent recovery for 24 h. We found that Capa and DH44 mRNA expression increased significantly following desiccation stress (4.8- and 2.7-fold, respectively, compared with the nonstressed control groups; P < 0.0001), while LK and DH31 expression remained unchanged. Following cold exposure, Capa mRNA levels were up-regulated (> 5-fold compared with untreated controls; P < 0.0001), while cold exposure had no significant effect on LK, DH44 or DH31 expression. Surprisingly, LK mRNA levels were up-regulated following recovery after cold challenge (Fig. 6b).

4 DISCUSSION
Research into the fruit pest D. suzukii has intensified in line with the continued global expansion of this species and the associated increased economic costs incurred as a consequence of crop damage. Much of this interest has focussed on D. suzukii’s ability to tolerate climatic conditions specific to the new geographical areas it is invading, focussing particularly on overwintering behaviours and the species’ responses to cold and desiccation environmental stresses.42–44 Here, by comparing the thermal stress tolerance of our Canton-S D. melanogaster with that of D. suzukii, we show that D. melanogaster seems to survive at significantly lower temperatures than D. suzukii. However, these results were solely obtained from a single strain of each Drosophila species and one should be cautious about making species-level conclusions because of the considerable genetic variation in stress traits across different populations of D. melanogaster and D. suzukii and even between different genotypes obtained from the same population.45,46 Nevertheless, females of D. suzukii have an LLT50 of -5.5 °C, and therefore were capable, at least for a short time, of surviving temperatures below those previously observed, where survival was shown to be limited below temperatures of 10 °C.47,48

It should be noted that experiments were performed on individuals of both species maintained at temperatures > 20 °C, corresponding to summer conditions. Recent research has shown that D. suzukii is capable of producing a winter morph phenotype when the summer morph is reared at temperatures of ≤ 10 °C, and that such a phenotype may aid winter survival and explain why D. suzukii has proved successful in cold climates.43 However, knowledge of the thermotolerance and thermal physiology of this winter phenotype is currently limited. A recent study has revealed the winter phenotype to display enhanced cold tolerance when compared with the summer phenotype with regard to measurement of the LLT50, although reduced cold tolerance with regard to measurement of the supercooling point (the temperature of crystallisation) and with substantial mortality occurring above the supercooling point.42 The authors suggest that the winter phenotype may not invest in cryoprotectants to prevent freezing,
Control of desiccation and cold tolerance by *D. suzukii*

![Figure 5](image)

**Figure 5.** Desiccation and cold tolerance in *D. suzukii* and *D. melanogaster*. (a) Desiccation resistance was significantly higher in *D. melanogaster* compared with *D. suzukii* and in females compared with males for both species (*P* < 0.0001; log-rank test; *n* = 90 – 130 flies for each *Drosophila* species). (b) Phenotype of *D. suzukii* desiccated for 18 h and water loss for *D. melanogaster* and *D. suzukii* exposed to desiccation. A significant increase in water loss was seen in *D. suzukii* flies; *P* < 0.05. (c) Survival curves for *D. melanogaster* and *D. suzukii*. Cold tolerance was significantly higher in *D. melanogaster* compared with *D. suzukii*, while no sex effect was apparent. *Drosophila melanogaster* is indicated by the blue lines and *D. suzukii* by the red lines. For each species, females are indicated by the block line and males by the dashed line.

but instead may utilise mechanisms to survive chill injury. How the cold tolerances of *D. suzukii* and *D. melanogaster* differ when in their respective winter phenotypes is presently unknown. However, at least in their summer morphs, our lab-reared Canton-S *D. melanogaster* is significantly more cold tolerant than *D. suzukii*.

Previous studies have presented contradictory descriptions comparing levels of cold tolerance between the sexes in *D. melanogaster*, with some studies observing that males demonstrate enhanced cold tolerance and others the converse. To clarify this, we tested male and female populations for both *D. melanogaster* and *D. suzukii* and observed no significant difference in cold tolerance between the sexes for both species. This apparent discrepancy may be a consequence of variation in the indices applied to the individual cold tolerance assays, i.e. chill coma, supercooling point, lethal time, lethal temperature etc., and/or variance in experimental factors, e.g. the age of the flies or the temperature at which they are tested.

In keeping with our observation that *D. melanogaster* proved harder in response to cold stress, we also observed that *D. melanogaster* was significantly more tolerant to desiccation than *D. suzukii*. Indeed, *D. suzukii* proved highly sensitive to desiccation, with complete lethality occurring in both sexes after approximately 24 h of desiccation (as opposed to *D. melanogaster* which survived approximately twice as long). Our data suggest that this increased sensitivity to desiccation is a consequence of the observed higher level of fluid loss. The lack of any physiological response to lower basal secretion rates in *D. suzukii* is surprising, and, although the reduced rate of water loss in desiccated *Drosophila* may be caused by combined reduction in respiratory, cuticular, and
excretory water loss, it is possible that, given the unchanged \textit{D. suzukii} basal secretion during desiccation stress, the MTs may play an important role in water balance during desiccation resistance.

Interestingly, whilst no difference was apparent in the cold tolerance responses of males and females, a significant sexually dimorphic effect was observed in \textit{D. suzukii} during desiccation, with females exhibiting enhanced desiccation resistance, surviving for 8 h longer than males. This is supported by previous work which found female \textit{D. suzukii} to be significantly more desiccation-resistant.\cite{58,59} In insects, where sexual dimorphism occurs, males and females are known to differ in their desiccation resistance, with larger body size associated with enhanced desiccation resistance.\cite{60} However, this does not hold true for starvation tolerance, as \textit{D. melanogaster} males survived starvation longer than females, despite the smaller size of males.\cite{61}


diuretic neuropeptides that act on \textit{D. melanogaster} MTs to modulate fluid homeostasis have also been found to modulate stress tolerance. Roles for Capa, LK and DH44 neuropeptide signalling in cold and desiccation has been recently demonstrated in \textit{D. melanogaster}. Our data for \textit{D. suzukii} indicate the involvement of Capa and DH44 signalling in desiccation, while Capa signalling is also involved in response to cold stress, as the expression levels for both neuropeptides were increased in response to these stresses and returned to basal levels within the recovery period. Surprisingly, LK mRNA levels were up-regulated during the recovery period following a cold challenge, suggesting that LK neurons may release LK peptide during recovery from cold stress. As LK does have demonstrated roles in feeding behaviour\cite{62} and starvation tolerance\cite{63} and starvation can be an associated factor during winter survival, a potential novel role for LK in cold tolerance responses in \textit{D. suzukii} would be a useful subject for future investigations.\cite{64}

In \textit{Drosophila}, several peptide hormones have been identified as pivotal to regulating ion transport and fluid secretion in the MTs and gut.\cite{29,37} Here, we describe in the pest species \textit{D. suzukii} the neuropeptide sequences for Capa, LK, DH44 and DH31, the neuroendocrine cells that produce these neuropeptides, their processes and the potential neural network that affects water homeostasis (and also feeding behaviour), showing it to be similar to that of \textit{D. melanogaster}. To fully determine the importance of these neuropeptides and receptor genes in \textit{D. suzukii} biology, it will be necessary to develop functional genomic tools that are currently employed in \textit{D. melanogaster}. Germline transformation techniques and Clustered Regularly Interspaced Short Palindromic Repeats/Crispr-associated proteins (CRISPR/Cas9)-mediated mutagenesis have already been successfully deployed in \textit{D. suzukii}\cite{64} and this ability to manipulate gene expression using a reverse genetic approach has already proved to be a powerful tool for understanding gene function.\cite{65} Unravelling the molecular, physiological and behavioural processes controlled by neuropeptides during environmental stress may augment the development of potential routes for targeted pest control.

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