Journal of Pest Science (2019) 92:1123–1137
https://doi.org/10.1007/s10340-019-01100-0

ORIGINAL PAPER

Desiccation, thermal stress and associated mortality in *Drosophila* fruit flies induced by neuropeptide analogue treatment

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Received: 15 October 2018 / Revised: 14 February 2019 / Accepted: 21 February 2019 / Published online: 14 March 2019

Abstract

Drosophila suzukii is a serious pest of soft fruit worldwide. With the global over-dependence on broad-spectrum pesticides, a strong imperative exists for more environmentally friendly and targeted methods of control. One promising avenue involves employing synthetic neuropeptide analogues as insecticidal agents to reduce pest fitness. Neuropeptides, central to the regulation of physiological and behavioural processes, play a vital role in cold and desiccation survival. Building upon this, the current study investigated the effects of biostable kinin, the cardioacceleratory peptide CAP2b and pyrokinin (PK) analogues (the latter of which have previously displayed cross-talk with the capa receptor), on desiccation, starvation and cold stress tolerance of the pest, *D. suzukii*, and the closely related non-pest, *D. melanogaster*. Results demonstrated analogues of the superfamily (CAP2b and PK derived) significantly impacted survival of the target insect under conditions of desiccation stress. However, these peptides enhanced desiccation stress survival in relation to controls, suggesting that they may act as antagonists of the capa signalling pathway in the Malpighian tubules. Of particular note was the ability of analogues 1895 (2Abf-Suc-FGPRLa) and 1902 (2Abf-Suc-FKPRLa) to impact *D. suzukii* but not *D. melanogaster*. A focus on native *Drosophila* CAP2b/PK and kinin sequences in analogue development may yield pure agonists with diuretic action that may reduce desiccation stress survival in the pest flies. In highlighting the PRXamide neuropeptide superfamily more generally, and the structures of promising analogues more specifically, this research will feed the evolution of next-generation analogues and drive forward the development of neuropeptidomimetic-based agents.

Keywords CAP2b · Dromekinin · *Drosophila suzukii* · G-protein-coupled receptors · Insecticide · Peptidomimetics · Pyrokinin

Key message

- The employment of synthetic neuropeptide analogues represents a novel approach to target-specific and environmentally friendly pest control.
- Biostable kinin, CAP2b and pyrokinin (PK) analogues were developed and tested ex vivo in Malpighian tubule fluid secretion assays.
- Analogues were subsequently tested in vivo for an ability to reduce the fitness of a pest species (*Drosophila suzukii*) under a variety of stress conditions relative to a non-pest species (*Drosophila melanogaster*).
- Results highlight the structure of neuropeptides of the PRXamide superfamily as candidates in the drive for neuropeptidomimetic-based insecticidal agents.

Communicated by A. Biondi.

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Introduction

Drosophila suzukii Matsumura (Diptera: Drosophilidae) is a pest of soft fruits worldwide (Asplen et al. 2015) and a close relative of the model organism *D. melanogaster*. Its pest status is due to the female's use of a specialised serrated

ovipositor to deposit eggs in ripe pre-harvest soft fruit crops (Atallah et al. 2014; Hamby et al. 2016). Recent research effort has focused on management strategies to minimise further spread of *D. suzukii* and associated economic losses, with a focus on detection, tracking and trap and bait design (Landolt et al. 2012; Feng et al. 2018; Frewin et al. 2017; Huang et al. 2017), biological control (e.g. Becher et al. 2017; Garriga et al. 2018; Giorgini et al. 2018; Girod et al. 2018; Woltz et al. 2015), harvesting techniques (Leach et al. 2018) and the development of biopesticides (Fanning et al. 2018). However, current management programs still rely heavily on broad-spectrum insecticides (Desneux et al. 2007; Diepenbrock et al. 2017). With the negative effects of chemical insecticides well documented (Pimentel et al. 1992), a strong imperative exists to develop more target-specific and 'greener' methods of pest control.

One promising avenue of pest control research involves the development and employment of neuropeptide synthetic mimetics (Nachman 2009). Neuropeptides are regulatory peptides and, within the insects, have functional roles in growth and development, behaviour and reproduction, metabolism and homeostasis and muscle movement (Altstein and Nässel 2010). Neuropeptides and their receptors (G-protein-coupled receptors, GPCRs) offer promising targets in the development of a new generation of insecticidal agents that selectively reduce the fitness of target pest insects, whilst minimising detrimental environmental impacts (Nachman 2009; Van Hiel et al. 2010; Audsley and Down 2015). Fundamental to this is an enhanced understanding of ligand–GPCR interactions to aid in the design of neuropeptide-based insecticidal agents.

Three neuropeptide families were selected for study including the insect cardioacceleratory peptides (capa, CAP2b; Kean et al. 2002), the pyrokinins (PK; Holman et al. 1986) and the kinins (Holman et al. 1999). Neuropeptides of the capa family were first identified in the moth *Manduca sexta* (CAP2b; Huesmann et al. 1995). Since their discovery, these neuropeptides have been shown to stimulate heart contractions (Huesmann et al. 1995), rapid fluid secretion by Malpighian (renal) tubules (Davies et al. 1995) and to modulate desiccation and cold tolerance in both *Drosophila melanogaster* and *D. suzukii* (Terhzaz et al. 2015). Widely distributed among invertebrates, the capa peptides belong to the PRXamide superfamily that can be further subdivided into three major classes: capa peptides, pyrokinins (PK) and ecdysis triggering hormone (ETH). Capa peptides may be defined by a characteristic C-terminus FXPRVamide.

The second family, the pyrokinins (PK), feature a characteristic C-terminus FXPRLamide and belong to the FXPRLaminde (pyrokinin/PBAN) family and the PRXamide superfamily (Altstein et al. 2013). First identified in the cockroach *Leucophaea maderae*, PK was found to be myotropic, activating the cockroach hindgut (Holman et al. 1986). However, the subsequent isolation of PKs in other invertebrates has led to some confusion over nomenclature with peptides featuring the characteristic C-terminus FXPR-Lamide being named after their functions, e.g. pheromone biosynthesis-activating neuropeptides (PBAN) and diapause hormone (DH), which are N-terminally extended pyrokinins found primarily in Lepidoptera, with specific neuronal and hormonal functions (DiNER; Yeoh et al. 2017). Pyrokinins are known to have roles in insect development, mating, muscle contraction and tannin (Altstein et al. 2013).

The final group, the insect kinins are multifunctional neuropeptides sharing a conserved C-terminal pentapeptide motif $FX^1X^2WGamide$, where X^1 can be Histidine, Asparagine, Serine or Tyrosine, and X^2 can be Serine, Proline or Alanine (Holman et al. 1999). Since their discovery, kinins have been identified in most insects including *D. suzukii* (Terhzaz et al. 2017) with the exception of Coleoptera (Halberg et al. 2015), and with roles in myostimulation (Holman et al. 1987; Schoofs et al. 1993), fluid secretion by the Malpighian tubules (Coast et al. 1990; Radford et al. 2002; Dow 2009; Coast et al. 2011), the release of digestive enzymes (Harshini et al. 2002, 2003) and inhibition of larval weight gain (Nachman et al. 2003; Seinsche et al. 2000).

In *Drosophila*, tolerance to cold and desiccation stress is modulated by gene expression for key physiological responses including ion transport, carbohydrate metabolism, antioxidants, immunity, signalling and gene expression pathways (Davies et al. 2014; Terhzaz et al. 2015). Recent work has also identified neuroendocrine signalling by kinin and capa peptides as a control mechanism of environmental stress tolerance, with the Malpighian tubules representing the primary target of neuropeptide action (Terhzaz et al. 2012, 2015, 2017; Cannell et al. 2016). Here, capa acts to increase intracellular calcium (Ca^{2+}), triggering a nitric oxide/cGMP signalling pathway in the tubule's principal cells (Rosay et al. 1997; Kean et al. 2002), whilst kinin acts on tubule stellate cells to elevate intracellular calcium (Ca^{2+}) and chloride shunt conductance (Terhzaz et al. 1999; Radford et al. 2002; Cabrero et al. 2014). Unlike capa and kinin, PKs are not known to have roles in environmental stress tolerance. However, cross-activity of PK analogues has been observed, with analogues displaying activity on recombinant capa receptors of *Tribolium castaneum* (Jiang et al. 2014, 2015). For this reason, selected PK analogues have been included in the current study into *Drosophila* desiccation and thermal stress, notably 1895 (Table 1) which exhibited agonist activity, and 1896 and 1902 (Table 1) which exhibited antagonist activity, on the *T. castaneum* TcCAPAr (Jiang et al. 2015). Furthermore, the addition of hydrophobic moieties to the N-terminus of PRXamide analogues, as exhibited by analogues 1895, 1896 and 1902 (Table 1), results in greater in vivo biostability (Zhang et al. 2011). Analogues 2089 and 2129 (Table 1) were subsequently designed and

Table 1 CAP2b, pyrokinin and kinin analogues

Code	Structure
CAP2b/PK	
1895	**2Abf-Suc-**FGPRLa
1896	**2Abf-Suc-**FTPRIa
1902	**2Abf-Suc-**FKPRLa
2089	**2Abf-Suc-**FTPRVa
2129	**2Abf-Suc-A**TPRIa
Kinin	
1728	**[Aib]**FF**[Aib]**WGa
2139	FF**[Aib]**WGa
2139-AC	Ac-FF**[Aib]**WGa

Modifications are shown in bold

synthesised as second-generation analogues of 1895, 1896 and 1902 to be evaluated in the current study. For the purpose of this study, these peptide analogues belonging to the PRXamide superfamily will be collectively referred to as 'CAP2b/PK'.

In addition to the Cap2b/PK analogues, kinin analogues were included in the current study due to their role in environmental stress control. However, insect kinins are subject to rapid biological degradation by peptidases. Incorporation of the α-amino isobutyric acid (Aib) at the third position of the insect kinin active core has been shown to protect the primary hydrolysis site from tissue-bound peptidase (Nachman et al. 1997a, b, 2002; Taneja-Bageshwar et al. 2006, 2009). Incorporation of a second Aib residue adjacent to the secondary peptidase hydrolysis site further enhances biostability (Nachman et al. 2002). Indeed, kinin analogues incorporating sterically bulky Aib residues adjacent to the primary and secondary hydrolysis sites have been shown to retain potent activity on receptors of the southern cattle tick, *Rhipicephalus* (Boophilus) *microplus* (Taneja-Bageshwar et al. 2006, 2009), and the dengue vector, the mosquito *Aedes aegypti* (Taneja-Bageshwar et al. 2006, 2009). For this reason, three biostable Aib analogues of the insect kinins 1728, 2139 and 2139-Ac were evaluated in the current study (Table 1).

The current study therefore aimed to elucidate the effect of biostable CAP2b/PK and kinin analogues on the stress tolerance of the pest insect *D. suzukii* and the closely related non-pest species *D. melanogaster*. Biostable analogues (Nachman 2009) were tested ex vivo for their physiological role in modulating fluid secretion via the Malpighian tubules and in vivo for their effect on desiccation, starvation and cold stress tolerance. To add additional validity to the screening process, promising analogues were further tested in vitro for their ability to trigger a Ca^{2+} signalling response in *Drosophila* S2 cells heterologously expressing prospective target receptors of the tested peptide analogues.

Material and methods

Drosophila rearing

Drosophila melanogaster wild-type flies were obtained from Bloomington Stock Centre and maintained on a standard *Drosophila* medium at 22 °C, 45–55% relative humidity and a 12:12 h light: dark photoperiod. *D. suzukii* flies were reared on standard blueberry-cornmeal agar medium, at 26 °C, 60% humidity with a 12:12 h light: dark photoperiod (Terhzaz et al. 2017). Adult *Drosophila* of both sexes were used in all experiments, 6–7 days after eclosion.

Neuropeptide analogue synthesis

The synthesis of neuropeptide analogues has been previously described as follows: CAP2b/PK analogues (displaying prior cross-talk with the capa receptor) 1895 and 1902 (Zhang et al. 2011; Jiang et al. 2015); 1896 (Jiang et al. 2015), 2089 and 2129 (using the method described in Zhang et al. 2011); kinin analogues 1728, 2139 and 2139-Ac (Taneja-Bageshwar et al. 2006, 2009). Analogues were purified and identity confirmed as detailed in Alford et al. (2019). The structures of the biostable analogues are displayed in Table 1.

Fluid secretion assay

Prior to in vivo screening, peptide analogues were first tested ex vivo for efficacy via a Ramsay fluid secretion assay for *Drosophila* Malpighian tubules (Dow et al. 1994; Terhzaz et al. 2017). Capa-1 at 10^{-5} M stimulates fluid secretion in *D. melanogaster* tubules (Kean et al. 2002) and calcium increase via the *capaR* (Terhzaz et al. 2012), but at levels which are not significantly different from 10^{-7} M. For this reason, analogues were first tested at a concentration of 1×10^{-5} M to efficiently rule out those analogues which failed to impact Malpighian tubule fluid secretion. Analogues that stimulated fluid secretion at 10^{-5} M were subsequently tested at lower concentrations down to 1×10^{-9} M. Briefly, Malpighian tubules from 6-d-old adult *D. melanogaster* and *D. suzukii* flies of mixed sex were isolated in Schneider's medium and suspended in a 9 µl drop of a 1:1 mixture of Schneider's medium: *Drosophila* saline. Male and female, posterior and anterior tubules were used interchangeably and grouped together due to showing no prior significant difference in secretion rate, with 10–15 secreting tubules per condition. Basal secretion rates were measured every 10 min for a period of 30 min. 1 µl of neuropeptide analogue (1×10^{-4} M stock solution) was then added to each well, resulting in a final concentration of 1×10^{-5} M. Secretion rates were subsequently measured for

an additional 30 min. Resultant data were analysed using a *t* test or Mann–Whitney *U* test to compare pre-stimulated basal time points (pooled 10–30 min) to each post-stimulated secretion time point. All analogues which stimulated fluid secretion at the supraphysiological dose of 1×10^{-5} M were subsequently tested at increasingly lower concentrations down to 1×10^{-9} M to test for differential sensitivity of the Malpighian tubules.

Microinjection of *Drosophila*

Native neuropeptides were diluted in *Drosophila* injection saline (Terhzaz et al. 2015) to 1×10^{-5} M. Neuropeptide analogues were diluted in *Drosophila* injection saline to the following concentrations : kinin analogues 1728 (2.5×10^{-5} M), 2139 (3.5×10^{-5} M), 2139-Ac (3.5×10^{-5} M); CAP2b/PK analogues 1895 (3.5×10^{-5} M), 1896 (3.5×10^{-5} M), 1902 (3.5×10^{-5} M), 2089 (3.9×10^{-5} M), 2129 (2.0×10^{-5} M). Neuropeptide solutions were administered to test flies at a sex-adjusted injection volume of 69 nL for females and 41 nL for males (Terhzaz et al. 2015). Injections were performed using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, Pennsylvania). Flies were injected in the thoracic segment below the left haltere. A vehicle control was set up on each treatment day. For this, control flies were injected with 69 nL or 41 nL of injection saline and exposed to identical conditions as flies receiving the neuropeptide treatment. Amaranth dye (Sigma; A1016) was added to all injected media to assist in visualising the success of an injection (i.e. to ensure that fluid was injected below the insect cuticle). Neuropeptide-treated and vehicle control flies were subsequently used in the stress bioassays detailed below.

Desiccation tolerance bioassay

Drosophila suzukii and *D. melanogaster* 6-d-old male and female flies were treated with a neuropeptide analogue or injection saline (vehicle control) via microinjection as detailed above. Following injection, flies were placed in groups of ten in 30 mL empty vials (no food or water) or vials containing 1% agar (no food) and the open end of the tube sealed with a cotton plug (buzzplug, Scientific Laboratory Supplies or Fly stuff, Flugs (49-103) (Bemis, NA)). Vials containing 1% agar provided flies with a water source and acted as the experimental control group. Surviving flies were counted every 3–4 h until no living flies remained. All experiments were run in triplicate with 20–30 flies for each species/sex/neuropeptide treatment combination. Survival data were subsequently analysed using a log-rank (Mantel–Cox) test in GraphPad Prism version 7.0.

Cold tolerance bioassay

Calculation of discriminating temperatures

The method for peptide analogue screening was adapted from the methodology commonly used to detect the presence of a rapid cold hardening response in insects (Lee et al. 1987). Here, variation in survival is compared between treatment groups at a predetermined 'discriminating temperature' (e.g. Powell and Bale 2005). To calculate a discriminating temperature, survival curves were first established for male and female *D. suzukii* and *D. melanogaster* to enable calculation of species-specific discriminating temperatures for subsequent neuropeptide testing. For this, adults of *D. suzukii* and *D. melanogaster* were selected at 6-d-old post-eclosion and exposed to a range of low temperatures (− 10 °C to − 2 °C at 1 °C intervals) using a direct plunge method (Sinclair and Chown 2006; Terblanche et al. 2008). 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 CO_2 and placed within plastic 0.5 mL 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, Germany) preset to the desired temperature. Pieces of cotton wool were used to stopper the boiling tubes to limit air circulation and to ensure a more stable internal temperature within the tubes. Adults were held at the desired exposure temperature for 1 h. Following exposure, adults were allowed to recover at the culture temperature in vials containing a food source and survival was assessed after 48 h. The procedure was repeated for each exposure temperature. A total of 270 male *D. melanogaster*, 270 female *D. melanogaster*, 240 male *D. suzukii* and 240 female *D. suzukii* were used to independently assess the cold tolerance of each species.

Survival data were analysed by Probit analysis in MINITAB, version 17 (Minitab Inc., State College, Pennsylvania), and the LT_{30} (the lethal temperature resulting in 30% mortality of a test population) was elucidated for each species. The LT_{30} was chosen to act as a discriminating temperature for subsequent neuropeptide testing since it enabled detection of directional effects of subsequent neuropeptide treatment, but primarily in the direction of interest, i.e. which neuropeptides significantly increased mortality in the species of interest. A separate discriminating temperature was calculated for males and females of each species to ensure that exposure to the discriminating temperature posed a comparable level of thermal stress for each species/sex treatment group.

Neuropeptide treatment and testing at the discriminating temperature

Individuals of *D. suzukii* and *D. melanogaster* were selected at 5-d-old post-eclosion and treated with neuropeptide analogues using the microinjection method detailed above. Following microinjection treatment, individuals were returned to vials containing food at densities of approximately 20–30 per vial and allowed to recover for 24 h at the culture temperature. Following the 24-h recovery period, adults of *D. suzukii* and *D. melanogaster* were cold shocked at the discriminating temperature following the same protocol as used to establish the discriminating temperature. Statistical analyses were performed using R software (R Development Core Team 2013). A generalised linear model (GLM) with binomial family was fitted to survival data with analogue 'Treatment' (peptide analogue), treatment 'Type' (test vs. control) and analogue treatment × treatment type interaction as factors.

Measurements of analogue-induced intracellular Ca^{2+} signalling response

Promising analogues from in vivo assays were taken forward and tested in vitro for an ability to trigger an intracellular Ca^{2+} signalling response in *Drosophila* S2 cells (Terhzaz et al. 2012). Transient transfection was performed with Effectene Transfection Reagent (Qiagen) using 1 µg of DNA of inducible pMT-CapaR and pMT-Apoaequorin expression vectors prepared according to manufacturer's instructions. Cells were plated in 6-well plates (1×10^6 cells/mL) and were incubated with the transfection mixture for 24 h before adding $CuSO_4$ (final concentration of 500 µM) to the cell culture (3 mL) for 48 h to induce *capaR* and Apoaequorin protein expression. Transfected S2 cells were harvested and incubated with 2.5 µM coelenterazine in the dark at room temperature for 1 h (Radford et al. 2002). Approximately 25,000 cells were subsequently placed in 175 µL Schneider's medium containing 10% FCS within a 5 mL Röhren tube (Sarstedt, Germany). Bioluminescence was recorded every 0.1 s using a Lumat LB 9507 luminometer (Berthold Technologies). Of a peptide analogue, 25 µL of a peptide analogue was applied to a final concentration of 10^{-7} M. Peptides were applied via the reagent rapid injectors of the Lumat LB 9507 luminometer into the aequorin-transfected S2 cell samples. This enables rapid and continuous recording of luminescence of the intracellular capa-induced Ca^{2+} signalling response (Rosay et al. 1997; Terhzaz et al. 2012). CAP2b (and capa) causes a rapid (< 100 ms) rise of $[Ca^{2+}]i$ that remains significantly above background for 90 s. The detection limit of the instrument has a time resolution of 100 ms; it may be that the actual response is even more rapid (Rosay et al. 1997). Accurate quantification of calcium levels at any point in the experiment requires the total available luminescence (i.e. the amount of reconstituted aequorin) to be known, since only a small fraction is used during the experiment (Cobbold and Rink 1987). Therefore, after a 5-min recording period, tissues were disrupted in 200 µL lysis solution (1% (*v*/*v*) Triton X-100, 100 mM $CaCl_2$), causing discharge of the remaining aequorin and allowing estimation of the total amount of aequorin in the sample by integration of total counts. The $[Ca^{2+}]$ concentration was subsequently calculated as previously described in Rosay et al. (1997).

Results

Fluid secretion assay

Ramsay secretion assays were performed on both *D. melanogaster* and *D. suzukii*, (Fig. 1). All three kinin analogues (1728, 2139 and 2139-Ac) tested at a concentration of $\times 10^{-5}$ M acted to significantly increase secretion rate (Fig. 1a–c). Of the CAP2b/PK neuropeptide analogues tested to a final concentration of $\times 10^{-5}$ M, four had no effect on Malpighian tubule secretion of either species: 1896, 1902, 2089 and 2129. CAP2b/PK analogue 1895 caused a small but significant decrease in the fluid secretion of *D. suzukii* (Fig. 1d), but not in *D. melanogaster*.

Lower concentrations of the three kinin neuropeptide analogues were tested to determine fluid secretion response of the Malpighian tubules of *D. melanogaster* and *D. suzukii* to these analogues. For analogue 1728, a final concentration of 2.5×10^{-7} M failed to elicit a response in the fluid secretion rate of the Malpighian tubules of either species (data not shown). Application of 2139 at 3.5×10^{-7} M caused a significant increase in the fluid secretion rate of the Malpighian tubules of *D. suzukii* but not *D. melanogaster* (Fig. 1e). Application of 2139 at the lower concentration of 3.5×10^{-8} M failed to elicit a response in *D. suzukii* (data not shown). Analogue 2139-Ac caused a significant increase in both species at 3.5×10^{-7} M (Fig. 1f), but failed to do so at 3.5×10^{-9} M (data not shown).

Desiccation stress

Results of the desiccation bioassay are displayed in Table 2, with selected survival curves shown in Fig. 2. Of the tested analogues, kinin analogue 2139 and CAP2b/PK analogue 1896 acted to significantly reduce survival under conditions of desiccation stress for *D. melanogaster*, although the effect was not consistent between the sexes. Here, 2139 significantly impacted only female survival (median survival: control 27.0 h, treatment 21.0 h) (Fig. 2a). 1896 significantly impacted only male survival, although median survival was

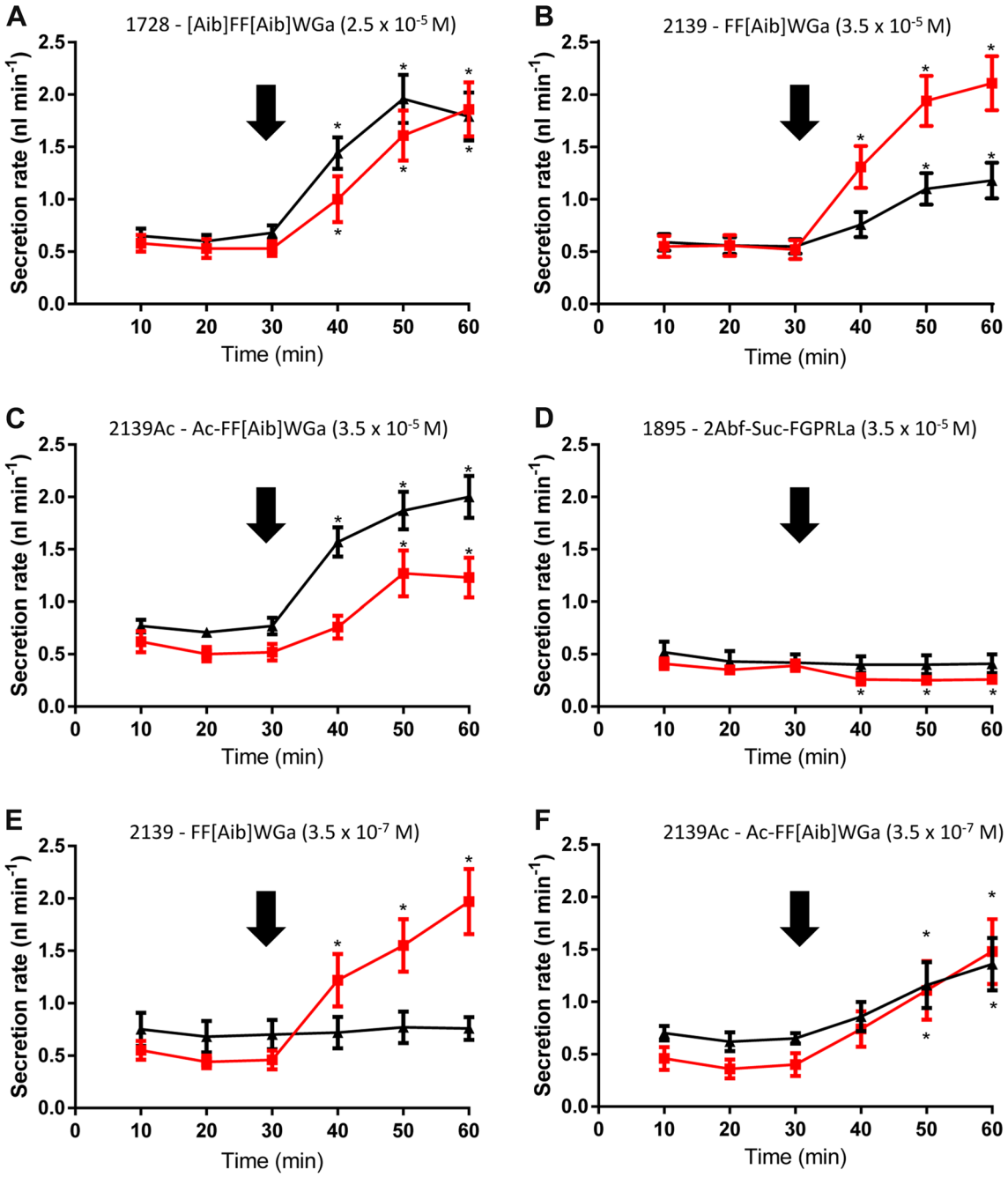

Fig. 1 Effects of neuropeptide analogues on fluid secretion rates of *D. melanogaster* (black) and *D. suzukii* (red) Malpighian tubules. Data show the response of tubule fluid secretion rate to **a** kinin 1728 (2.5×10^{-5} M), **b** kinin 2139 (3.5×10^{-5} M), **c** kinin 2139-Ac (3.5×10^{-5} M), **d** CAP2b/PK 1895 (3.5×10^{-5} M), **e** kinin 2139 (3.5×10^{-7} M) and **f** kinin 2139-Ac (3.5×10^{-7} M). Tubules were stimulated with the analogue at 30 min, as indicated by the black arrow. Data are presented as mean ± SEM; $p<0.05$

identical between the control and treatment group, (median survival: control 14.0 h, treatment 14.0 h) (Fig. 2b). Interestingly, 2139 had no effect on the desiccation tolerance of *D. suzukii* (Fig. 2a), and 1896 acted to significantly increase the survival of female *D. suzukii* (median survival: control 20.5 h, treatment 23.5 h) (Fig. 2b). CAP2b/PK analogues 1895 and 1902 (Fig. 2b) significantly increased survival of both male and female *D. suzukii* under desiccation stress (1895 median survival: male control 8.0 h, treatment 10.0 h; female control 16.0 h, treatment 25.5 h) (1902 median survival: male control 7.0 h, treatment 14.0 h; female control 17.0 h, treatment 21.0 h), although showed no effect on *D. melanogaster* survival. Analogue 2129 significantly improved female *D. melanogaster* (median survival control:

Table 2 (a) CAP2b, pyrokinin and kinin analogue impact on desiccation tolerance of male and female *D. melanogaster* and *D. suzukii*. (b) Experimental control flies were subjected to a starvation stress in the presence of a water source (1% agar). Survival is shown as median survival (h) ± interquartile range

(a)

	Desiccation					
Peptide	Male	Median survival ± IQR (h) vehicle control|treatment		Female	Median survival ± IQR (h) vehicle control|treatment	
D. melanogaster						
Kinin			*p*			*p*
1728		22.0 ± 3.0|22.0 ± 0.0	0.579		22.0 ± 2.0|22.0 ± 0.0	**0.004**
2139		20.0 ± 3.0|20.5 ± 3.0	0.614		27.00 ± 23.3|21.0 ± 6.0	**< 0.001**
2139-AC		11.0 ± 13.0|11.0 ± 10.0	0.488		8.0 ± 14.3|16.0 ± 13.0	**0.024**
CAP2b/PK						
1895		18.0 ± 9.0|18.0 ± 9.1	0.082		29.5 ± 7.0|31.5 ± 12.0	0.113
1896		14.0 ± 5.0|14.0 ± 4.0	**0.016**		25.0 ± 9.0|25.0 ± 14.8	0.573
1902		19.0 ± 14.0|19.0 ± 7.8	0.136		33.0 ± 15.0|29.0 ± 12.0	0.228
2089		25.0 ± 12.5|33.0 ± 11.0	0.375		39.0 ± 14.0|37.50 ± 18.0	0.655
2129		19.0 ± 15.8|19.0 ± 15.0	0.879		14.0 ± 12.0|27.00 ± 15.0	**< 0.001**
D. suzukii						
Kinin			*p*			*p*
1728		6.0 ± 3.0|6.0 ± 6.3	0.939		20.0 ± 0.0|20.0 ± 0.0	0.802
2139		7.0 ± 17.0|8.0 ± 17.0	0.198		22.0 ± 0.0|22.0 ± 0.0	0.832
2139-AC		3.0 ± 4.00|6.0 ± 3.0	**< 0.001**		7.5 ± 9.0|19.0 ± 4.0	**< 0.001**
CAP2b/PK						
1895		8.0 ± 7.0|10.0 ± 8.0	**0.010**		16.0 ± 13.5|25.50 ± 6.3	**< 0.001**
1896		8.5 ± 4.0|8.5 ± 4.0	0.706		20.5 ± 7.0|23.5 ± 9.0	**0.009**
1902		7.0 ± 0.8|14.0 ± 7.0	**< 0.001**		17.0 ± 7.8|21.0 ± 10.0	**0.034**
2089		13.0 ± 7.6|15.0 ± 7.8	0.291		23.0 ± 11.0|23.0 ± 8.0	0.550
2129		5.0 ± 6.0|6.5 ± 15.0	**0.029**		17.0 ± 5.0|24.0 ± 7.0	**< 0.001**

(b)

	Starvation control					
Peptide	Male	Median survival ± IQR (h) vehicle control|treatment		Female	Median survival ± IQR (h) vehicle control|treatment	
D. melanogaster						
Kinin			*p*			*p*
1728		47.0 ± 0.0|47.0 ± 16.5	0.177		74.0 ± 21.8|74.0 ± 6.0	0.283
2139		47.0 ± 0.0|47.0 ± 0.0	0.304		*|*	NA
2139-AC		39.0 ± 11.5|35.0 ± 15.3	0.876		*|*	NA
CAP2b						
1895		64.5 ± 33.8|76.5 ± 24.3	0.789		97.0 ± 36.3|107.5 ± 45.5	0.143
1896		56.0 ± 14.3|75.0 ± 26.3	**0.008**		83.0 ± 45.8|132.0 ± 50.0	**0.037**
1902		68.0 ± 28.0|62.0 ± 22.3	0.219		80.0 ± 8.5|80.0 ± 19.5	0.985
2019		85.0 ± 21.3|93.0 ± 22.0	0.538		142.0 ± 23.0|127.0 ± 39.0	0.102
2129		53.0 ± 18.5|63.0 ± 26.3	0.191		71.0 ± 23.8|88.0 ± 55.3	0.058
D. suzukii						
Kinin			*p*			*p*
1728		30.0 ± 13.0|25.0 ± 5.8	0.734		48.5 ± 27.0|48.0 ± 6.0	**0.035**
2139		23.0 ± 6.0|22.0 ± 4.8	0.282		49.0 ± 0.0|49.0 ± 0.0	0.737
2139-AC		26.0 ± 7.0|19.0 ± 14.0	**0.018**		*|*	NA
CAP2b						
1895		34.5 ± 13.3|36.5 ± 17.0	0.406		75.5 ± 33.5|78.5 ± 12.0	0.899
1896		33.5 ± 17.0|33.5 ± 11.0	0.170		52.5 ± 13.0|57.5 ± 61.0	0.476
1902		32.5 ± 16.0|32.5 ± 15.0	0.626		69.0 ± 21.0|66.0 ± 15.0	0.675
2019		52.0 ± 5.3|55.0 ± 11.3	0.208		85.0 ± 34.8|85.0 ± 21.0	0.321
2129		32.5 ± 17.0|32.0 ± 19.1	0.316		81.5 ± 34.0|79.0 ± 26.5	0.811

Table 2 (continued)

Significant effects are shown in bold. Selected survival curves are displayed in Fig. 2

* analysis not possible due to low n numbers

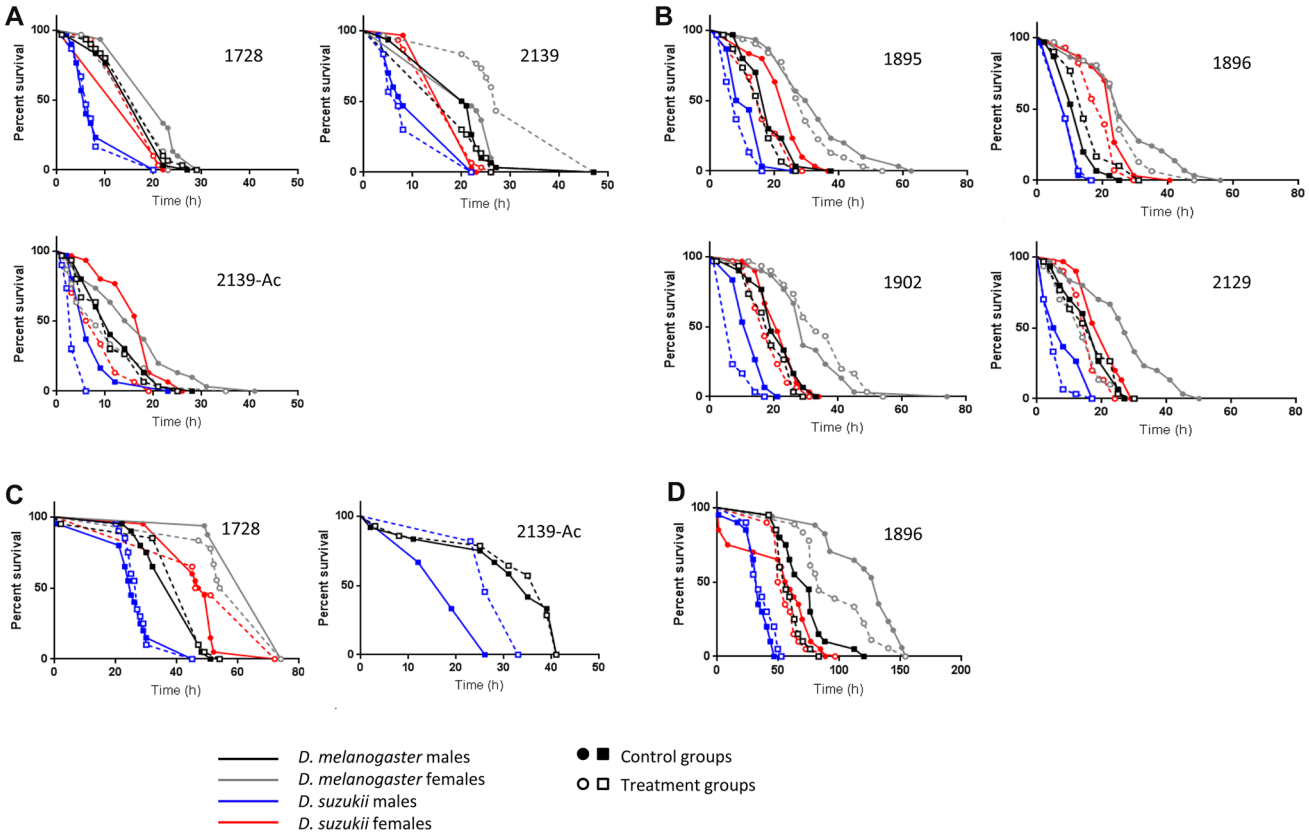

Fig. 2 Selected survival curves of *Drosophila melanogaster* males (black), *D. melanogaster* females (grey), *D. suzukii* males (blue) and *D. suzukii* females (red) when treated with biostable peptide analogues via microinjection and subjected to either desiccation stress or starvation stress. **a** Kinin analogue treatment under desiccation stress; **b** CAP2b/PK analogue treatment under desiccation stress; **c** Kinin analogue treatment under starvation stress; **d** CAP2b/PK analogue treatment under starvation stress. Treatment groups are indicated by the block line and vehicle control groups by the dashed line

14.0 h, treatment 27.0 h) and both male and female *D. suzukii* survival (median survival: male control 5.0 h, treatment 6.5 h; female control 17.0 h, treatment 24.0 h) under desiccation stress, whilst male *D. melanogaster* was unaffected by 2129 (Fig. 2b).

The effect of analogue treatment under the experimental control condition of starvation stress in the presence of a water source (1% agar) was minimal. Kinin analogue 1728 acted to decrease female *D. suzukii* survival, but with enhanced mortality only occurring after the point of $LTime_{50}$ (i.e. the time taken to kill 50% of the test population) (median survival 48.5 and 48.0 h for the control and treatment group, respectively) (Fig. 2c). In contrast, treatment with 2139-Ac decreased male survival under conditions of starvation stress (median survival: control 26.0 h, treatment 19.0 h) (Fig. 2c). The kinin analogues had no significant effect on the starvation tolerance of *D. melanogaster*. Of the CAP2b/PK analogues, only 1896 significantly increased survival of both male and female *D. melanogaster* (median survival: male control 56.0 h, treatment 75.0 h; female control 83.0 h, treatment 132.0 h) (Fig. 2d). The CAP2b/PK analogues had no significant effect on the starvation tolerance of *D. suzukii*.

Cold stress

Survival curves were calculated for *D. melanogaster* and *D. suzukii* of both sexes (Fig. 3), and the LT_{30} (discriminating temperature) was calculated as follows: *D. melanogaster* females − 6.7 °C; *D. melanogaster* males − 6.8 °C; *D. suzukii* females − 4.9 °C; and *D. suzukii* males − 4.5 °C.

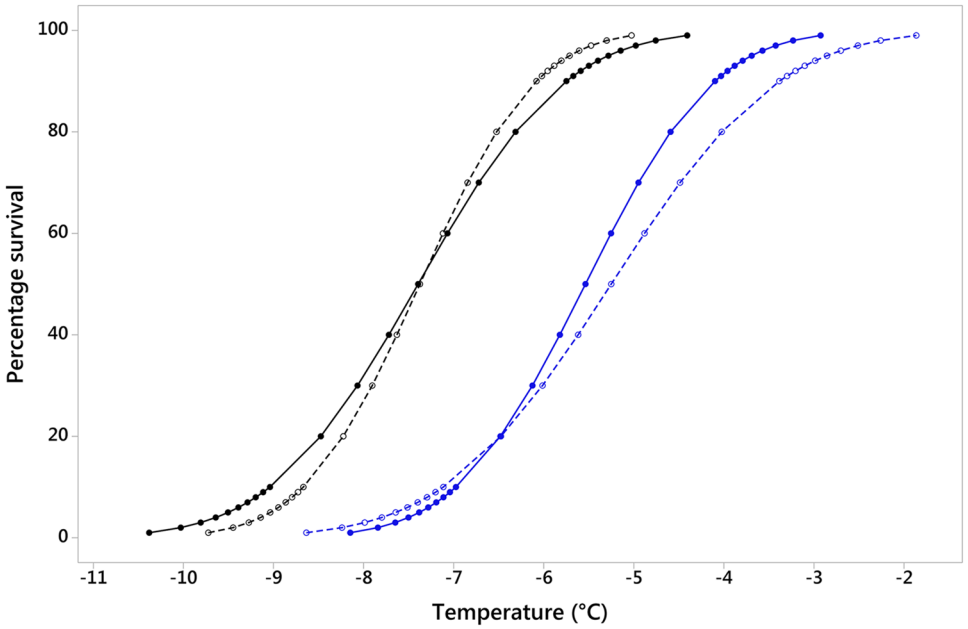

Fig. 3 Survival curves calculated via Probit analysis of *D. melanogaster* (black symbols) and *D. suzukii* (blue symbols) following a 1-h exposure at the desired temperature. For both species, males are indicated by the open symbol and dashed line and females by the solid symbol and the solid line

Calculated discriminating temperatures were used for subsequent neuropeptide screening.

Results of the desiccation and starvation bioassay revealed kinin analogues 1728, 2139 and 2139-Ac and CAP2b/PK analogues 1895 and 2129 to be of most interest in eliciting an effect on survival. These neuropeptides were subsequently carried forward for use in the cold stress bioassay.

There was a significant effect of 'Type' (control vs. treatment) on the cold stress survival of *D. melanogaster* males following cold shock at the discriminating temperature (GLM DF = 1, $\chi^2 = 34.931$, $p = 0.018$), indicating that all analogues are increasing fly survival relative to control groups under conditions of cold stress (Fig. 4). However, there was no effect of the factor 'Treatment' (peptide analogue + associated control) on male *D. melanogaster* cold stress survival (GLM DF = 4, $\chi^2 = 32.714$, $p = 0.696$), indicating that all analogues appear equivalent in their effect, with no analogue having a stronger effect than another. In contrast, there was no effect of 'Type' on the cold stress survival of *D. melanogaster* females (GLM DF = 1, $\chi^2 = 84.293$, $p = 0.723$), *D. suzukii* females (GLM DF = 1, $\chi^2 = 126.745$, $p = 0.729$) and the survival of *D. suzukii* males (GLM DF = 1, $\chi^2 = 44.505$, $p = 0.543$) when subjected to their discriminating temperature, indicating that peptide analogue treatment failed to impact survival (Fig. 4).

Intracellular Ca^{2+} response

Due to eliciting a response in vivo in the desiccation stress assays, the CAP2b/PK analogues were carried forward for in vitro testing for agonistic properties employing transfected *Drosophila* S2 cells expressing prospective target receptors of the tested peptide analogues. Stimulation of the capa receptor (*capaR*) with capa-1 results in a biphasic rise in intracellular Ca^{2+}, constituting a rapid primary peak followed by a gradual secondary peak (Fig. 5a) (*cf* Terhzaz et al. 2012). Stimulation with CAP2b/PK analogue 1895 initiated a Ca^{2+} response (Fig. 5b), although to a lower magnitude than that observed for the native capa-1 neuropeptide. Stimulation with CAP2b/PK analogues 1896, 1902, 2089 and 2129 failed to initiate a Ca^{2+} response, suggesting that the analogues are not acting on the *capaR* (in Fig. 5c, only data for 1896 are shown to represent a non-response). Analogue 1895, which had elicited a stimulatory response against the *capaR* receptor, failed to initiate a response when tested against the *Drosophila* pyrokinin 2 (PK2) receptor (Terhzaz et al. 2012) (Fig. 6b). In contrast, 1896 (Fig. 6c) and 1902 (Fig. 6d) both initiated a Ca^{2+} response when tested on S2 cells expressing the *Drosophila* PK2 receptor, with 1902 producing a response similar in magnitude to the signature biphasic response observed for native pyrokinin (Fig. 6a) (Terhzaz et al. 2012), suggesting that these analogues act as ligands for the PK2 receptor but not *capaR*.

Discussion

The neuropeptidergic system offers a promising target for the development of novel, environmentally friendly insecticidal agents and over the last decade has received increasing research attention (Kaczmarek et al. 2010; Smagghe et al. 2010; Zhang et al. 2011; Nachman et al. 2011). Key to the development of peptidomimetic-based insecticides is

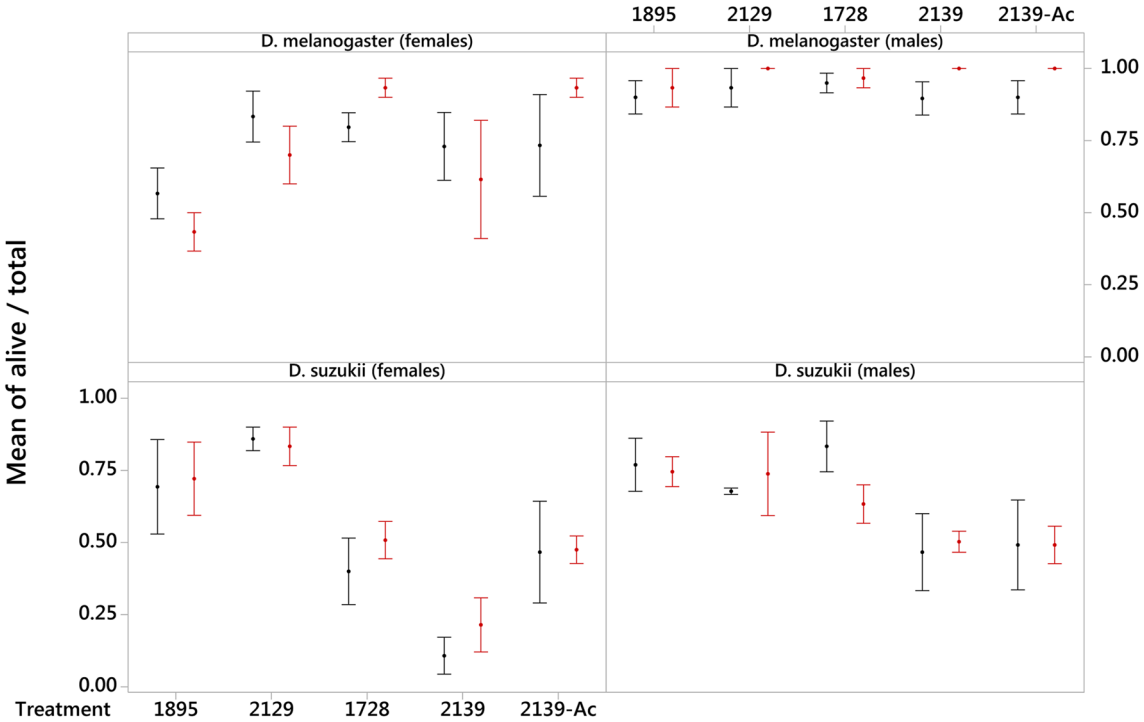

Fig. 4 Mean ± SE proportion survival of *D. melanogaster* females, *D. melanogaster* males, *D. suzukii* females and *D. suzukii* males when treated with biostable peptide analogues (CAP2b/PK: 1895, 2129; kinin: 1728, 2139, 2139-Ac) via microinjection and subjected to a discriminating temperature for a 1-h exposure. Control groups are shown in black and peptide treatment groups in red

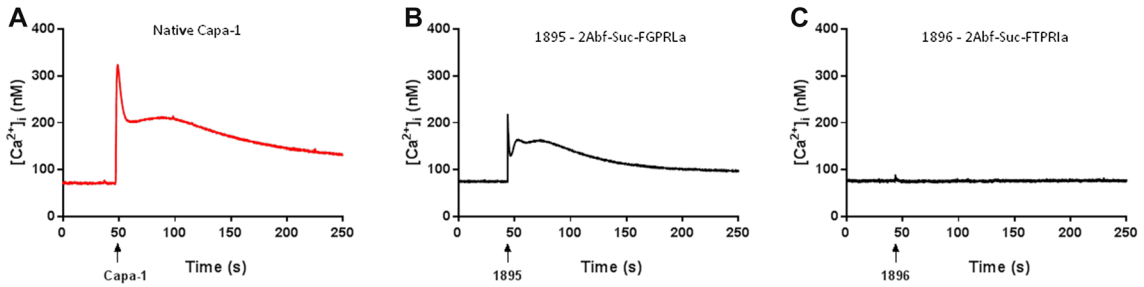

Fig. 5 Capa and biostable analogue-associated calcium signatures. **a** Typical cytoplasmic Ca^{2+} response in S2 cells expressing the *capaR* and apoaequorin challenged with capa-1 at a concentration of 10^{-7} M. Cytoplasmic Ca^{2+} response in S2 cells when challenged with analogues, **b** 1895 and **c** 1896 (representative of a non-response) at a concentration of 10^{-5} M

the screening of potential biostable analogues for species-specific effects. The current study assayed five biostable CAP2b/PK analogues of the PRXamide superfamily and three biostable kinin analogues for an ability to reduce pest fitness under conditions of desiccation, starvation and thermal (cold) stress, employing *D. suzukii* as a target pest insect and the closely related *D. melanogaster* as a non-pest insect.

Of the screened analogues, all kinin analogues (1728, 2139, 2139-Ac) acted to increase the fluid secretion rate of *D. melanogaster* and *D. suzukii* at concentrations of $\times 10^{-5}$ M, in agreement with previous work showing that kinin neuropeptides are diuretic, stimulating rapid fluid secretion in the Malpighian tubules of *D. melanogaster* and *D. suzukii* (Terhzaz et al. 1999, 2017). Furthermore, when testing kinin analogues at increasingly lower concentrations, analogue 2139 stimulated fluid secretion in *D. suzukii* at concentrations of $\times 10^{-7}$ M, but failed to elicit a response in *D. melanogaster*, suggesting enhanced sensitivity of the *D. suzukii* kinin receptor to analogue 2139. Although all the kinin analogues acted to increase the fluid secretion rate, this did not correlate to a consistent directional effect on *D. melanogaster* or *D. suzukii* survival under conditions of desiccation stress. The reason for this is not known, although one explanation may be due to in vivo receptor desensitisation

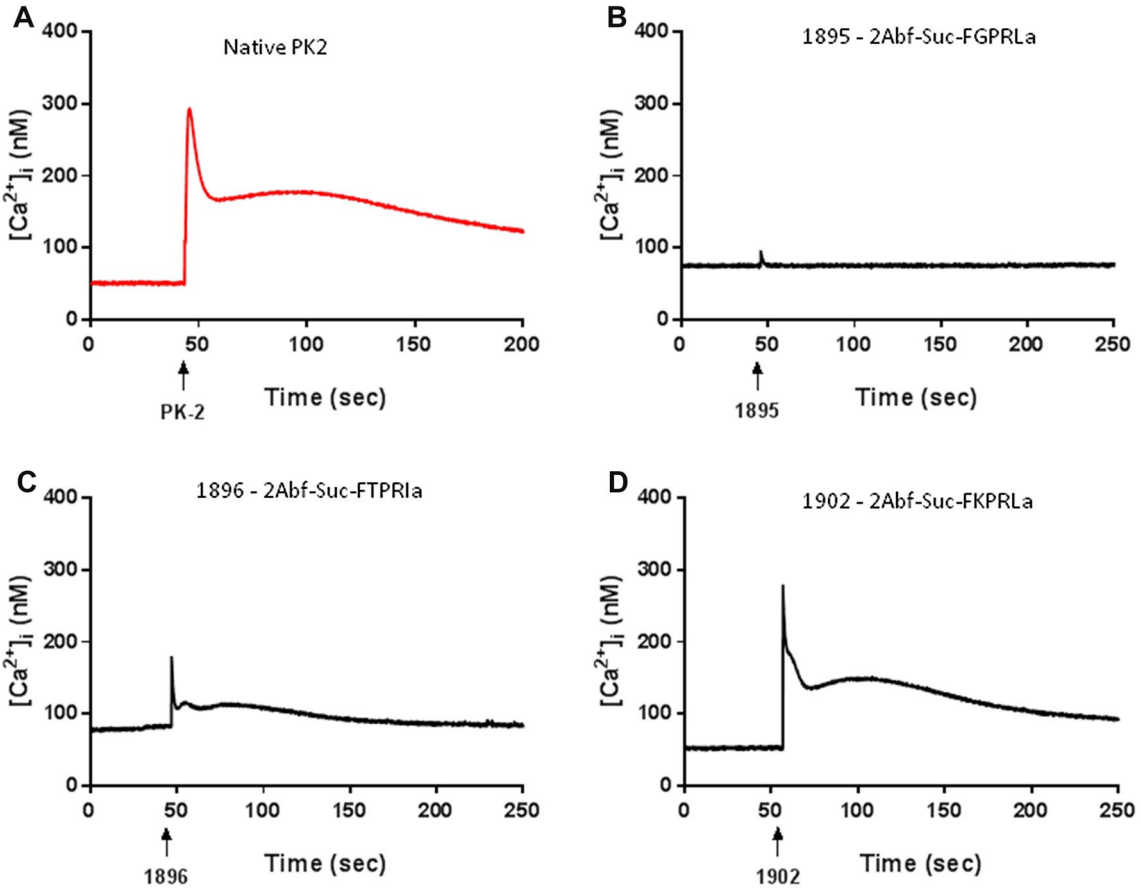

Fig. 6 Pyrokinin and biostable analogue-associated calcium signatures. **a** Typical cytoplasmic Ca^{2+} response in S2 cells expressing the PK2 receptor and apoaequorin challenged with pyrokinin 2 at a concentration of 10^{-7} M. Cytoplasmic Ca^{2+} response in S2 cells when challenged with analogues, **b** 1895, **c** 1896 and **d** 1902 at a concentration of 10^{-5} M

under the conditions and time frame of the desiccation stress assay. Furthermore, in the current study, which investigated *Drosophila* stress tolerance, the Malpighian tubules were the desired target of analogue action. Direct injection of the analogues into the haemolymph was thus chosen as the mode of application to maximise the probability of injected analogues reaching the target organ. However, we cannot exclude analogue action on the *Drosophila* CNS and further work perturbing the kinin signalling system is required to elucidate this (Zandawala et al. 2018).

In contrast to the kinin analogues, none of the CAP2b/PK analogues screened significantly affected the rate of fluid secretion in the Malpighian tubules of either *D. melanogaster* or *D. suzukii*, with the exception of 1895 which resulted in a small decrease in the secretion of *D. suzukii* tubules. It is known that neuropeptides of the capa family have roles in the stimulation of fluid secretion, acting via elevation of intracellular calcium and activation of the NO-cGMP signalling pathway (Davies et al. 1997; Kean et al. 2002). This failure of most of the screened CAP2b analogues to impact fluid secretion, either as a diuretic or an anti-diuretic, suggests an inability of the analogues to bind to *capaR* and initiate a calcium signalling response. This was supported via screening of the CAP2b analogues in *Drosophila* S2 cells which were tested for agonistic properties, affirming an inability of analogues 1896, 1902, 2089 and 2129 to initiate a calcium response. Only analogue 1895 stimulated *capaR* to produce a calcium response although this was significantly lower in magnitude for both primary and secondary peaks compared to the capa-induced *capaR* response, Fig. 5a (Terhzaz et al. 2012).

Drosophila melanogaster encodes three putative pyrokinin receptors: CG9918, PK1-R; CG8795, PK2-R2; CG8784, PK2-R1 (Flybase, Grimmelikhuijzen, C.J.P. (2013.6.6). Nomenclature of Pyrokinin receptor-encoding genes). The previous work has shown that *D. melanogaster* PK1 (capa-3 TGPSASSGLWFGPRLamide) and PK2 (renamed PK-gamma, SVPFKPRLamide) and hugin activate calcium signalling via CG8795, PK2-R2 (Choi et al. 2003; Terhzaz et al. 2012). Analogue 1895 has previously been shown to interact with the *Tribolium* PK receptor (Jiang et al. 2015), and analogue 1902 also contains PK-like motifs

(PRLamide). In addition, 1896 contains the PRIamide motif, which is also seen, for example, in *H. abietis* PK-1 (DiNER; Yeoh et al. 2017). Therefore, 1895, 1896 and 1902 were assessed against the functional PK receptor encoded by CG8795. 2129 was not assessed against the PK receptor, as the critical F residue in the PK core FXPRLa, which is required for PK receptor interaction, is replaced with an 'A' in 2129. Analogue 2129 also possesses steric hindrance adjacent to the alpha carbon in the C-terminal position (e.g. as found with Ile or Val) which is strongly preferred for binding to *capaR* but which will interfere with PK receptor binding.

More recently, peptides of the capa family have been linked to desiccation and cold tolerance in *Drosophila*, with the capability (capa) neuropeptide gene found to be desiccation and cold stress responsive (Terhzaz et al. 2015). In addition, knock-down of the capa gene has been shown to increase *Drosophila* desiccation tolerance (Terhzaz et al. 2015). In the current study, it was the CAP2b/PK analogues that showed the most promise in significantly impacting survival under conditions of desiccation stress, whilst causing minimal effects under conditions of starvation stress. Here, analogues 1895, 1896, 1902 and 2129 significantly impacted female *D. suzukii* desiccation survival and 1895, 1902 and 2129 male *D. suzukii* desiccation survival, whilst having no (1895, 1902) or limited (1896, 2129) effect on *D. melanogaster*. These selected neuropeptides offered a protective effect and thus increased survival under desiccation stress. Furthermore, when under conditions of cold stress, all analogues produced a protective effect in *D. melanogaster* males, although failed to impact the survival of female *D. melanogaster* and both male and female *D. suzukii*. The reason for this male *D. melanogaster*-specific effect is unknown. According to the absolute energy demand (AED) hypothesis, larger bodied individuals are hypothesised to be at a disadvantage when under stressful conditions such as thermal stress due to expending energy at a proportionately faster rate than smaller bodied individuals (Reim et al. 2006). Under this hypothesis, smaller bodied *D. melanogaster* males would be considered at an advantage when under cold stress. However, why analogue treatment would significantly affect only *D. melanogaster* male cold stress survival is unknown.

As desiccation survival is tightly linked with fluid secretion by Malpighian tubules, the lack of effect on fluid secretion by many of the analogues tested may explain the desiccation survival phenotypes upon treatment with analogues 1896, 1902 and 2129. The protective effect of these CAP2b analogues may also be due to an antagonist response against binding of endogenous capa neuropeptides to *capaR* in vivo, as both 1896 and 1902 demonstrated an antagonist effect on the heterologous *T. castaneum* TcCAPAr (Jiang et al. 2015), although further testing is required to confirm this. The inability of both 1896 and 1902 to initiate a Ca^{2+} response in the S2 cell assay when targeting the *capaR* adds support to this. 1896 and 1902 instead showed activation of the pyrokinin receptor, which has not been shown to influence fluid secretion rates (Kean et al. 2002). These findings may indicate a, heretofore unknown, role for PK, and the PK receptor, in desiccation survival.

The current study investigated the effect of biostable kinin and CAP2b/PK analogue treatment on *Drosophila* stress tolerance (desiccation, starvation and cold temperature stress), focusing on *D. suzukii* as the target (pest) species and *D. melanogaster* as a non-target species. Of the kinin and CAP2b/PK analogues, it was the CAP2b/PK analogues which displayed the most promise in altering the relative fitness of treated *D. suzukii* when under conditions of desiccation stress. Of particular interest were analogues 1895 (2Abf-Suc-FGPRLa), 1896 (2Abf-Suc-FTPRIa), 1902 (2Abf-Suc-FKPRLa) and 2129 (2Abf-Suc-ATPRIa), with 1895 and 1902 showing increased promise due to an ability to target the pest *D. suzukii*, whilst leaving the non-pest *D. melanogaster* unaffected.

In order to develop neuropeptide analogues that can reduce survival in pest fruit flies, future directions in analogue design should focus on the inclusion of *D. suzukii* peptide sequences (DiNER; Yeoh et al. 2017), as well as the addition of polyethylene glycol (PEG) polymer moieties that can increase bioavailability characteristics (Boccù et al. 1982; Jeffers and Roe 2008). Testing of new-generation analogues is currently underway with a focus on mode of application to elucidate the most efficacious method of delivery to apply neuropeptide-based insecticides in the field.

Author contributions

LA, RM, JATD and SAD conceived and designed the research. RJN designed and produced peptide analogues. LA and RM conducted experiments with assistance from AD. LA and RM analysed data. LA wrote the manuscript, and RM, RJN, SAD and AD edited it. All authors read and approved the manuscript.

Funding This work was funded by the European Union's Horizon 2020 Research and Innovation programme under grant agreement No.634361 (SD/JATD) (nEUROSTRESSPEP) and the US Department of Agriculture/Department of Defence Deployed War Fighter Protection Initiative 6202-22000-029-00D (RJN).

Compliance with ethical standards

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

Ethical approval The article does not contain any studies with human participants or animals (vertebrates).

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