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**Assessment of neuropeptide binding sites and the impact of biostable kinin
and CAP2b analogue treatment on aphid (*Myzus persicae* and
Macrosiphum rosae) stress tolerance**

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Running title:

Aphid stress tolerance and the development of neuropeptide-based insecticidal agents

Abstract

BACKGROUND: Neuropeptides are regulators of critical life processes in insects and, due to their high specificity, represent potential targets in the development of greener insecticidal agents. Fundamental to this drive is understanding neuroendocrine pathways that control key physiological processes in pest insects and the screening of potential analogues. The current study investigated neuropeptide binding sites of kinin and CAPA (CAPA-1) in the aphids

Myzus persicae and *Macrosiphum rosae* and the effect of biostable analogues on aphid fitness under conditions of desiccation, starvation and thermal (cold) stress.

RESULTS: *M. persicae* and *M. rosae* displayed identical patterns of neuropeptide receptor mapping along the gut, with the gut musculature representing the main target for kinin and CAPA-1 action. Whilst kinin receptor binding was observed in the brain and VNC of *M. persicae*, this was not observed in *M. rosae*. Furthermore, no CAPA-1 receptor binding was observed in the brain and VNC of either species. CAP2b/PK analogues (with CAPA receptor cross-activity) were most effective in reducing aphid fitness under conditions of desiccation and starvation stress, particularly analogues 1895 (**2Abf-Suc-FGPRLa**) and 2129 (**2Abf-Suc-ATPRIa**), which expedited aphid mortality. All analogues, with the exception of 2139-Ac, were efficient at reducing aphid survival under cold stress, although were equivalent in the strength of their effect.

CONCLUSION: In demonstrating the effects of analogues belonging to the CAP2b neuropeptide family and key analogue structures that reduce aphid fitness under stress conditions, this research will feed into the development of second generation analogues and ultimately the development of neuropeptidomimetic-based insecticidal agents.

Keywords

Aphicide, Cold Tolerance, Desiccation, Ligand-Binding, G-Protein Coupled Receptors
Receptor-Mapping, Pest Control

1. Introduction

With a global dependence on broad-spectrum insecticides, the damaging effects of which are well documented,^{1,2} there is increasing need for the development of greener, target-specific insecticides. The development and employment of neuropeptide synthetic analogues offers a promising avenue in the drive for greener and target-specific insecticidal agents. Within the insects, neuropeptides are regulatory peptides with functional roles in growth and development, behaviour and reproduction, metabolism and homeostasis, and muscle movement.³ Due to their high specificity, neuropeptides and their cognate receptors (G-protein coupled receptors, GPCRs) may be developed as an insecticidal target system^{4,5} to selectively reduce the fitness of target pest insects, whilst minimising detrimental environmental impacts.

The two neuropeptide families selected for study include the insect kinins and CAP2/CAPA peptides. Insect kinins are multifunctional neuropeptides which share a conserved C-terminal pentapeptide motif Phe-X¹-X²-Trp-Gly-NH₂, where X¹ can be His, Asn, Ser or Tyr, and X² can be Ser, Pro or Ala.⁶ The insect kinins have been identified in most insects, with the exception of Coleoptera,⁷ and have diverse roles in the stimulation of muscle,⁸ fluid secretion in renal tubules,^{9,10} digestive enzyme release,¹¹ inhibition of larval weight gain¹² and the desiccation and starvation stress response.^{13,14} The second family, the CAPA peptides, were first identified from the moth *Manduca sexta* (CAP2b)¹⁵ and have since been identified in many insect families.¹⁶ Although function varies depending on insect species, life stage, and lifestyle, CAPA peptides play a key role in myomodulation and osmoregulation¹⁶ and have more recently been linked to desiccation and cold tolerance in *Drosophila* species.^{17,18}

The CAPA peptides belong to the PRXamide superfamily which can be further subdivided into three major classes: CAPA peptides, pyrokinins (PK) and ecdysis triggering hormone (ETH).¹⁹ The pyrokinins are further subdivided into diapause hormone (DH) and pheromone biosynthesis activating neuropeptides (PBAN) and by their C-terminal motifs WFGPRLamide and FXPRLamide respectively.²⁰ The GPCRs of this ligand group form a homologous cluster, suggesting co-evolution of ancestrally related ligand-receptor partners. As a result, some cross activity by analogues of the ligand sub-groups with respective, recombinant receptors has been observed.^{21,22} For this reason, certain PK analogues that have previously demonstrated cross activity on recombinant CAPA receptors of *Tribolium castaneum* have been included in this study. In particular, analogue 1895 (Table 1) has exhibited agonist activity, and analogues 1896 and 1902 (Table 1) antagonistic activity on *T. castaneum* TcCAPAr.²² Furthermore, PRXamide analogues with the addition of hydrophobic moieties at the N-terminus have been shown to display greater biostability *in-vivo*,²³ as featured in 1895, 1896 and 1902. Subsequently, 2089, 2123, 2125 and 2129 (Table 1) were designed and synthesized as second generation analogues of 1895, 1896 and 1902 to be evaluated in the current study.

Insect kinins are prone to rapid degradation by peptidases within the insect haemolymph and bound to tissues. The angiotensin converting enzyme (ACE) from the housefly is capable of cleaving insect kinin at the primary hydrolysis site, and neprilysin (NEP), at both the primary and secondary hydrolysis sites,²⁴⁻²⁸ thus representing biostability issues. In order to overcome this issue, incorporation of a single α -amino isobutyric acid (Aib) into the third position of the insect kinin active core protects the primary hydrolysis site from tissue-bound peptidase.²⁶⁻³⁰ Incorporation of a second Aib residue adjacent to the secondary peptidase hydrolysis site further enhances biostability.²⁶ Insect kinin analogues that incorporate

sterically-bulky Aib residues, adjacent to both primary and secondary peptidase hydrolysis sites were previously evaluated on two recombinant receptor assays from the southern cattle tick, *Rhipicephalus (Boophilus) microplus*,^{31,32} and the dengue vector, the mosquito *Aedes aegypti*.³³ These results demonstrated that biostable Aib analogues of the insect kinins can retain potent activity on these two receptors.³⁰

Recent studies have documented the potent effects of biostable neuropeptide analogues on pest insects.^{30,34,35} By employing two aphid pest species, kinin³⁶ and CAPA³⁷ receptor sites were first mapped in aphid tissue. The potential effects of kinin and CAP2b/PK analogues on aphid stress tolerance and fitness (desiccation, starvation, cold) were subsequently screened, representing the first study into the role of these neuropeptide families in aphid stress tolerance. Aphids (Hemiptera: Aphididae) are one of the most significant groups of agricultural pests³⁸ and are vectors in the transmission of approximately 50% of all insect transmitted plant viruses.³⁹ The primary study species for the current study, the peach potato aphid *Myzus persicae*, is the most economically important aphid crop pest worldwide,⁴⁰ with a global distribution and host range encompassing more than 400 species in 40 different plant families.⁴¹ The secondary study species, the rose aphid *Macrosiphum rosae*, was selected to represent a major pest of horticulture. *M. rosae* is an important pest of cultivated species of *Rosa* and is a vector in the transmission of 12 plant viruses including the strawberry mild yellow edge virus.⁴¹ The results of this study will inform design and development of novel, specific insecticidal agents.

2. Material and methods

2.1 Aphid rearing

Stock cultures of anholocyclic *M. persicae* were established using aphids supplied by the Smaghe laboratory, Ghent University, Belgium. Cultures were reared under a 12:12 h LD photocycle at 22°C on Chinese cabbage (*Brassica rapa* var. Wong Bok) contained within a BugDorm fine mesh cage (44545F) (45cm x 45cm x 45 cm). A fresh supply of Chinese cabbage of approximately 4 weeks from sowing was supplied to the cages on a once-weekly basis to maintain the aphid cultures.

M. rosae was selected as a secondary aphid species and a sub-set of experiments was performed on the species to determine the overlap in response between aphid species of different genera. Stock cultures of anholocyclic *M. rosae* were set up from individual aphids originally collected on *Rosa* species within the grounds of the University of Glasgow, Scotland, UK. A stock culture was set up within the laboratory and maintained on supermarket-bought miniature rose plants and under identical conditions to *M. persicae*. Prior to use, the leaves of the rose plants were thoroughly washed using 70% ethanol followed by distilled water to ensure the removal of any potential chemical residues present on the plant.

2.2 Neuropeptide synthesis

Native and fluorescently labelled neuropeptides CAPA-1 (CAPA-1-F) and kinin (Kinin-F) were synthesized by Cambridge Peptides (Birmingham, UK) as previously detailed⁷ and based on the CAPA and kinin structures of *Drosophila melanogaster*. In brief, native kinin was synthesized and coupled to Alexafluor488 resulting in fluorescent kinin (Alexa-488-C₅-maleimide-CNSVVLGKKQRFHSWGamide). The same rationale was used for the production of CAPA-1 (GANMGLYAFPRVamide) and labelled CAPA-1-F with the

addition of TMR-C₅-Maleimide Bodipy dye (TMR-C₅-maleimide-CGANMGLYAFPRVamide).

The synthesis of PK analogues (with CAPA receptor cross-activity) 1895 and 1902,^{22,23} CAP2b analogue 1896,²² and insect kinin analogues 1728 and 2139^{29,30} have been previously described. CAP2b analogues 2089, 2123, 2125, and 2129;²³ as well as insect kinin analogue 2139-Ac²⁹ were synthesized and cleaved according to procedures that have been previously described. The analogues were purified on a Waters Delta-Pak C18 reverse-phase column (8 x 100 mm, 15 µm particle size, 100 Å pore size) with a Waters 510 HPLC system with detection at 214 nm at ambient temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); Solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Initial conditions were 10% B followed by a linear increase to 90 % B over 40min.; flow rate, 2 ml/min. Delta-Pak C18 retention times: 2089, 12.0 min.; 2123, 9.0 min; 2139-Ac, 5.9 min; 2125, 12.5 min; 2129, 7.5 min. The analogues were further purified on a Waters Protein Pak I 125 column (7.8 x 300 mm). Conditions: isocratic using 80% acetonitrile containing 0.1% TFA; flow rate, 2 ml/min. Waters Protein Pak retention times: 2089, 6.0 min; 2123, 5.5 min; 2139-Ac, 5.9 min; 2125, 5.5 min; 2129, 6.0 min. Amino acid analysis was carried out under previously reported conditions (Nachman et al., 2004) to quantify the analogues and to confirm identity: 2089: F[1.0], P[1.0], R[1.0], T[1.0], V[1.0]; 2123: F[1.0], R[0.9], T[0.9], V[0.9]; 2139-Ac: F[2.0], G[0.9]; 2125: F[1.0], R[0.8], T[0.7], V[0.8]; 2129: A[1.0], I[0.9], P[0.9], R[0.9], T[0.9]. The identity of the analogues was also confirmed by MALDI-MS on a Kratos Kompact Probe MALDI-MS instrument (Shimadzu, Columbia, Maryland). The following molecular ions (MH⁺) were observed: 2089, 961.0 (calc.961.8); 2123, 976.2 (calc.976.1); 2139-Ac, 704.7 (calc.704.5, [MNa⁺]); 2125, 1014.1 (calc.1014.0); 2129, 898.8 (calc.898.8). The structures of the biostable analogues are displayed in Table 1.

2.3 Receptor mapping assay using fluorescently labelled neuropeptides

Aphids were cold anesthetised and the tissue of interest dissected out in a 1:1 solution of Schneider's insect medium and optimised saline.^{7,42} The dissected tissue was mounted on a poly-L-lysine-covered 35mm glass bottom dish containing 1:1 saline. Nuclei were stained via incubation in DAPI ($1 \mu\text{g ml}^{-1}$) for 1 minute and then washed with the optimised saline solution. A baseline image was taken to determine the level of autofluorescence and adjust exposure settings accordingly. All images were recorded on an inverted confocal microscope (Zeiss LSM 510 Meta). A labelled neuropeptide (10^{-7} M) was subsequently added to the tissue and the tissue incubated for 1 minute before washing with the optimised saline solution. The sample tissue was immediately imaged. The concentration of 10^{-7} M was chosen for labelled neuropeptides because it represents the minimal concentration required to produce a saturated receptor response, thereby optimising the conditions for optical detection of ligand-receptor complexes.⁷ Following imaging, unlabelled neuropeptide (10^{-5} M) was added to the sample and a time-lapse experiment set up to determine if the unlabelled neuropeptide outcompeted the labelled neuropeptide, thus affirming detection of the ligand-receptor complexes. Images were collected every 30 s for a duration of 20-30 m. All imaging was repeated on a minimum of three specimens to ensure consistency and further re-affirm conclusions. All images were exported as JPEG files and subsequently viewed in FIJI and Microsoft Illustrator. When specific binding was observed in muscle tissue, this was supported by the addition of rhodamine phalloidin; a high-affinity F-actin probe conjugated to tetramethylrhodamine (TRITC) that specifically binds to muscle.

2.4 Neuropeptide treatment via microinjection

Neuropeptides were administered to test aphids via microinjection to allow for rapid mass screening of neuropeptide analogue efficacy. For this, native neuropeptides were diluted in double distilled water (DDH₂O) to a concentration of 1×10^{-5} M. Neuropeptide analogues were diluted in DDH₂O 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), 2123 (1.0×10^{-5} M), 2125 (1.0×10^{-5} M), 2129 (2.0×10^{-5} M). Once at the desired concentration, neuropeptide solutions were administered to test aphids at an injection volume of 9nl based on total haemolymph volume, to produce an approximate 1:20 dilution of injection volume to haemolymph. Injections were performed using a pulled glass needle and a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Company, Broomall, Pennsylvania). A vehicle control was set up for each treatment / day of experiments to account for variation in needle pulling. For this, control aphids were injected with 9nl of DDH₂O and subsequently exposed to the same experiments as aphids receiving the neuropeptide treatment. Neuropeptide treated and vehicle control aphids were subsequently used in the stress bioassays detailed below.

2.5 Desiccation / starvation tolerance bioassay

Anholocyclic adults of mixed age of either *M. persicae* or *M. rosae* were selected from the stock cultures and treated with a native neuropeptide or neuropeptide analogue via microinjection using the method detailed above. Treated and vehicle control aphids were allowed to recover on excised leaves of the host plant for 1 h before being placed in an empty ventilated microcage (L = 4 cm, Ø = 9.5 cm) at densities of 10 per cage. In total, 30-40 aphids were treated for each neuropeptide treatment group and a further 30-40 for the associated vehicle control group (i.e. 3-4 biological replicates of 10). From the point of placement in the microcage (taken as 0 h), aphid survival was checked every hour during

daylight hours and approximately every 4 h during night-time hours until the final aphid died. Survival data were subsequently analysed using a Log-rank (Mantel-Cox) test in GraphPad Prism version 7.0. $LTime_{50}$ (the time taken to kill 50% of the test population) values were calculated via Probit Analysis in Minitab 17 (Minitab Inc., State College, Pennsylvania). Neuropeptide analogues which significantly impacted aphid survival under desiccation / starvation stress were further tested under non-stress conditions. For this, aphids were treated with the neuropeptide analogue as detailed above and maintained on excised leaves of the host plant for a duration of 7 days. Survival data at day 7 were arcsine square root transformed and analysed using analysis of variance (ANOVA) and Tukey's multiple range test in MINITAB version 17.

2.6 Cold tolerance bioassay

2.6.1 Calculation of discriminating temperatures

M. persicae and *M. rosae* displayed identical results in desiccation / starvation stress assays. For this reason, and given its global pest status, only *M. persicae* was taken forward in cold stress assays. Survival curves were first established to determine a species-specific discriminating temperature for subsequent neuropeptide testing. Aphids were selected at the pre-reproductive adult stage for cold tolerance bioassays since aphid cold tolerance is known to significantly vary throughout an aphid's life cycle.^{43,44} Temperature ranges were selected to encompass 0-100% mortality. Anholocyclic pre-reproductive adults (approximately 9 d old at 22°C) of *M. persicae* were exposed to a range of low temperatures (-14°C to -7°C at 1°C intervals) using a direct plunge method.^{45,46} For each temperature treatment, 30 adults were placed within plastic 0.5mL 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) pre-set 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, aphids were allowed to recover at the culture temperature in microcages containing excised leaves of the host plant and survival was assessed after 48 h. The procedure was repeated for each exposure temperature.

Survival data were analysed using 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. 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.

2.6.2 Peptide analogue treatment and testing at the discriminating temperature

Pre-reproductive anholocyclic adult aphids of *M. persicae* were treated with neuropeptide analogues using the microinjection method detailed above. Following microinjection treatment, individuals were returned to microcages containing excised leaves of the host plant at densities of approximately 20-30 per microcage and allowed to recover for 24 h at the culture temperature. Following the 24 h recovery period, adults were placed within plastic 0.5mL Eppendorf tubes at densities of ten adults per tube to a total of 30 for each species x neuropeptide treatment group (i.e. 3 biological replicates of 10 per replicate). Eppendorf tubes were then placed within glass boiling tubes held within the alcohol bath pre-set to the desired discriminating 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 microcages containing excised leaves of the host plant and survival was assessed after 48 h. The procedure was repeated for each species x peptide analogue treatment group.

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 x treatment type interaction as factors.

3. Results

3.1 Receptor mapping assay using fluorescently labelled neuropeptides

A fluorescent ligand-receptor binding assay was employed to map specificity of binding of Kinin and CAPA-1 within *M. persicae* and *M. rosae*. Fluorophore-labelled kinin (kinin-F) and CAPA-1 (CAPA-1-F) revealed the neuropeptides to bind to circular muscles along the aphid gut (Figure 1). Kinin-F binding to longitudinal muscles in the hindgut region of the gut was further suggested (data not shown). Both the kinin-F and CAPA-1-F signals were displaced by excess unlabelled peptide in the ligand competition assay, thus confirming specificity of binding. Additional labelling with rhodamine phalloidin acted to confirm the gut muscle as the site of binding (Figure 1c). Interestingly, specific kinin-F and CAPA-1-F binding of the gut musculature was not evident under low magnification (x10) (Figure 2). The presence of smaller cells, running the length of the gut, were detected as a site of kinin-F binding (Figure 1b and 2 indicated by white arrows), although were not a site of CAPA-1-F binding (Figure 1a). In addition, CAPA-1-F specific binding was detected in a region of the aphid midgut (stomach) closest to the foregut (Figure 3) and may represent enterocytes, although further testing is required to confirm this.

Receptor mapping of the *M. persicae* brain and ventral nerve cord (VNC) revealed kinin-F staining apparent in a bilateral symmetrical ‘ladder’ of neuronal clusters (2-3 neurons) and a set of baso-lateral neurons in the suboesophageal ganglion (Figure 4a). Staining was also apparent in symmetrical pairs of neurons/neuronal clusters in the ventro- to dorso-lateral protocerebrum. Little to no kinin-F staining was observed in the VNC with the exception of a set of cells in the most distal tip of the abdominal ganglion (Figure 4b). In contrast, no specific staining with kinin-F was observed in the brain or VNC of *M. rosae*. Labelling with CAPA-1-F revealed no sites of receptor binding in either the brain (Figure 4c) or the VNC of both species (Figure 4d).

3.2 Desiccation stress

The CAP2b analogues 1895 and 2129 significantly increased desiccation / starvation mortality in both species (Table 2, Figure 5). Here, treatment with 1895 acted to reduce the LTime₅₀ by 3.5 h and 9.6 h in *M. persicae* and *M. rosae* respectively, and median survival by 4.0 h and 10.5 h respectively (Table 2). Treatment with 2129 acted to reduce the LTime₅₀ by 7.1 h and 11.6 h in *M. persicae* and *M. rosae* respectively, and median survival by 9.8 h and 12.8 h respectively (Table 2). Neither 1895 nor 2129 impacted survival under non-stress conditions (ANOVA DF = 2 , F = 0.00, p = 0.999) with 1 out of 32 aphids treated with 1895, 1 out of 31 aphids treated with 2129, and 1 out of 29 aphids from the control group dying within 7 days post treatment. None of the kinin analogues significantly affected desiccation/starvation mortality in either species (Table 2).

3.3 Cold stress

A survival curve was calculated for *M. persicae* (Figure 6) and the LT₃₀ (discriminating temperature) calculated as -9.7°C. There was a significant effect of ‘Type’ (control vs. treatment) on the cold stress survival of *M. persicae* following cold shock at the discriminating temperature of -9.7°C (GLM DF = 1, $\chi^2 = 5.9844$, $p = 0.014$), indicating that all analogues are efficient at increasing the mortality of test aphids under conditions of cold stress. However, there was no effect of the factor ‘Treatment’ (peptide analogue) on aphid cold stress survival (GLM DF = 9, $\chi^2 = 7.8355$, $p = 0.551$), indicating that all analogues appear equivalent in their effect, with no analogue having a stronger effect than another. Interestingly, treatment with analogue 2139-AC implies a reverse effect on aphid survival (Figure 7), acting to increase survival relative to the control. However, further examination restricted to this case against its control proved non-significant (GLM DF = 1, $\chi^2 = 7.8355$, $p = 0.3771$). It must be concluded that all studied analogues increase the mortality of aphids under conditions of cold stress, with the exception of 2139-Ac, although no individual peptide is significantly more powerful in its effect.

4. Discussion

Neuropeptides are regulators of critical life processes in insects and, due to their high specificity, hold great potential in the drive for target-specific and environmentally friendly insecticidal agents.⁵ In pursuit of the development of neuropeptidomimetic-based insecticides, the current study mapped kinin (kinin-F) and CAPA (CAPA-1-F) neuropeptide binding sites within *M. persicae* and *M. rosae* to determine the location of neuropeptide receptors and thus the targets of neuropeptide action. A total of 10 kinin and CAP2b/PK (active on the CAPA receptor) biostable analogues were subsequently assayed for target-insect-specificity and an ability to reduce aphid pest fitness under a range of environmental stressors.

Receptor mapping employing fluorescently labelled kinin revealed the gut musculature as a main target for kinin activity in both *M. persicae* and *M. rosae*, as previously shown for *M. persicae*.⁷ Additional areas in the brain and VNC were also indicated in *M. persicae*. In the pea aphid, *Acyrtosiphon pisum*, it is thought that kinin regulates gut motility, digestive enzyme release, fluid cycling and nutrient transport across the gut.³⁴ Indeed, kinin analogues have shown great potential in the laboratory for their aphicidal properties, acting as antifeedant agents during artificial diet trials on the pea aphid (*A. pisum*).³⁴ Interestingly, whilst kinin analogues, including the present analogue 1728, have displayed prior antifeedant potential, none of the kinin analogues in the current study acted to reduce aphid fitness under desiccation and starvation stress conditions.

As with receptor mapping of kinin activity, receptor mapping with fluorescently labelled CAPA revealed the muscles of the aphid midgut as the target for neuropeptide binding, with no CAPA receptor binding detected in the aphid brain or VNC. However, in contrast to the kinin analogues, CAP2b/PK analogues displayed greater promise in stress tolerance assays, with analogues 1985 (**2Abf-Suc-FGPRLa**) and 2129 (**2Abf-Suc-ATPRIa**) acting to expedite aphid (*M. persicae* and *M. rosae*) mortality under conditions of desiccation and starvation stress. Furthermore, all tested analogues (kinin and CAP2b/PK), with the exception of 2139-Ac, enhanced *M. persicae* mortality under cold stress conditions, although were all considered equivalent in the strength of their effect.

Neuropeptides of the CAPA family have roles in the stimulation of fluid secretion in Malpighian (renal) tubules⁴⁷ and, more recently, have been linked to desiccation and cold tolerance in *Drosophila*.¹⁷ Unlike most insects, aphids lack Malpighian tubules;⁴⁸ organs with

vital roles in osmoregulation, detoxification and immunity.^{49,50} Due to this secondary loss of Malpighian tubules in the aphids, key osmoregulatory roles have been reassigned to other organs, particularly the aphid gut.⁵⁰ Receptor mapping assays performed in the current study offer support to this, highlighting the presence of CAPA receptors along the aphid gut and implicating the gut as a primary target for CAPA neuropeptide action. The role of CAPA neuropeptides in osmoregulation further offers explanation for the relative effectiveness of the CAP2b analogues tested in expediting aphid mortality under conditions of desiccation stress.

5. Conclusion

Kinin and CAPA (CAPA-1) binding was confirmed in the aphids *M. persicae* and *M. rosae*, with the musculature of the gut the primary target of neuropeptide action. *In-vivo* assays revealed CAP2b/PK analogues to be the most effective in expediting aphid mortality under conditions of desiccation and starvation stress. In highlighting the PRXamide neuropeptide family more generally, and the structures of promising CAP2b/PK analogues more specifically, this research will feed into the development of second and third generation analogues and ultimately drive forward the development of neuropeptidomimetic-based insecticidal agents. Current testing of new generation analogues is focusing on mode of application to elucidate the best method of delivery to apply neuropeptide-based insecticides in the field.

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Table 1 The structure of biostable CAP2b, pyrokinin (with CAPA receptor cross activity) and kinin analogues used in aphid stress tolerance assays. Modifications are shown in bold.

Code	Structure
CAP2b / PK	
1895	2Abf-Suc-FGPRLa
1896	2Abf-Suc-FTPRIa
1902	2Abf-Suc-FKPRLa
2089	2Abf-Suc-FTPRVa
2123	2Abf-Suc-FT[Hyp]RVa
2125	2Abf-Suc-FT[Oic]RVa
2129	2Abf-Suc-ATPRIa
Kinin	
1728	[Aib]FF[Aib]WGa
2139	FF[Aib]WGa
2139-AC	Ac-FF[Aib]WGa

Table 2 The effect of neuropeptide analogue treatment via microinjection on the desiccation and starvation tolerance of *M. persicae* and *M. rosae*. Neuropeptide analogues were administered to a final concentration of $\times 10^{-5}$ M. Survival is shown as both a median survival (h) \pm IQR and an LTime₅₀ (h). Values in bold significantly increased desiccation / starvation mortality in relation to a vehicle control group. Example survival curves are displayed in Figure 5.

<i>Myzus persicae</i>					
	Documented effect	LTime ₅₀ (h) control test	Median \pm IQR survival (h) control test	χ^2	p
1728	No effect	18.3 25.0	22.0 \pm 16.5 26.0 \pm 23.5	2.260	0.133
2139	No effect	18.3 22.4	22.0 \pm 16.5 25.0 \pm 21.0	1.341	0.247
2139-AC	No effect	19.4 16.7	22.5 \pm 19.8 20.0 \pm 14.0	2.498	0.114
1895	Increases mortality	15.9 12.4	17.8 \pm 12.0 13.8 \pm 11.8	3.948	0.047
1896	No effect	22.2 24.8	24.0 \pm 09.0 25.0 \pm 10.5	1.939	0.164
1902	No effect	22.2 21.8	24.0 \pm 09.0 23.5 \pm 07.0	0.030	0.863
2089	No effect	10.9 13.0	11.0 \pm 12.0 16.5 \pm 10.3	2.197	0.138
2123	No effect	24.3 21.1	25.0 \pm 13.5 21.0 \pm 13.5	2.092	0.148
2125	No effect	10.8 13.0	11.0 \pm 12.0 11.0 \pm 12.0	1.309	0.253
2129	Increases mortality	15.9 08.8	17.8 \pm 12.0 08.0 \pm 17.0	10.200	0.001
<i>Macrosiphum rosae</i>					
	Documented effect	LTime ₅₀ (h) control test	Median \pm IQR survival (h) control test	χ^2	p
1728	No effect	31.7 31.6	29.5 \pm 22.0 24.0 \pm 28.5	0.431	0.512
2139	No effect	31.7 29.8	29.5 \pm 22.0 27.0 \pm 26.0	0.176	0.675
2139-AC	No effect	44.0 40.1	39.0 \pm 37.0 35.0 \pm 32.0	0.210	0.647
1895	Increases mortality	18.7 09.1	17.5 \pm 19.5 07.0 \pm 12.5	14.060	<0.0001
1896	No effect	28.9 24.7	27.0 \pm 17.3 23.0 \pm 18.5	0.065	0.799
1902	No effect	28.9 26.4	27.0 \pm 17.3 25.0 \pm 10.0	0.224	0.636
2089	No effect	23.2 16.4	23.0 \pm 17.0 17.0 \pm 21.3	2.002	0.157
2123	No effect	21.6 19.1	24.0 \pm 11.0 16.0 \pm 18.0	1.806	0.179
2125	No effect	21.6 20.1	24.0 \pm 9.25 20.5 \pm 18.0	0.215	0.643
2129	Increases mortality	24.3 12.7	23.8 \pm 20.5 11.0 \pm 13.0	14.320	<0.0001

Figure 1 Aphid intestine (distal midgut and proximal hindgut) stained with 10^{-7} M CAPA-1-F labelled with TMR C5-Maleimide (A) shows receptor localisation in the gut muscles (*Myzus persicae* shown). Excess unlabelled CAPA-1 (10^{-5} M) displaces fluorescent signal in a ligand competition assay (not shown), thus confirming the specificity of binding. Aphid intestine stained with 10^{-7} M kinin labelled with alexafluor488 (B) shows receptor localisation in the gut muscles (*M. rosae* shown). Excess unlabelled kinin (10^{-5} M) displaces fluorescent signal in a ligand competition assay (not shown), thus confirming the specificity of binding. Staining by kinin-F was also present in a population of basal cells, characterised by smaller nuclei, as indicated by the white arrows. DAPI was used for nuclear staining (blue). Staining with rhodamine phalloidin labelled with tetramethylrhodamine (TRITC) reaffirms the gut musculature as the site of receptor binding (C). (A and B): Kinin-F, green; CAPA-1-F, red; DAPI, blue. (C) Kinin-F, green; rhodamine phalloidin, red; DAPI, blue. Scale bars = 20 μ m.

Scale Bars = 20 μ m

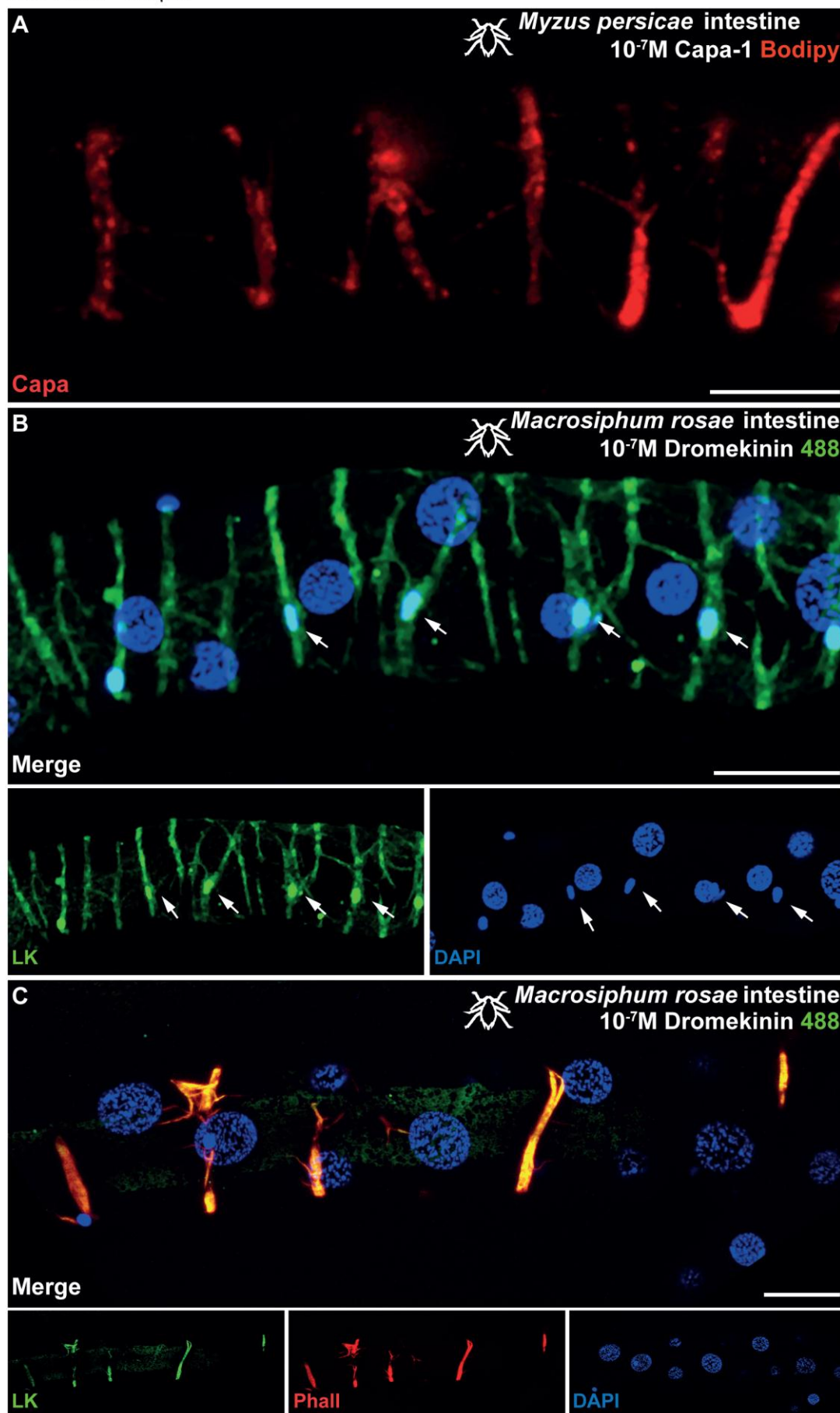


Figure 2 *Myzus persicae* intestine (distal midgut and proximal hindgut) stained with 10^{-7} M kinin labelled with alexafluor488 (A) and then out-competed with 10^{-5} M unlabelled kinin (B). (A) Staining apparent in a population of basal cells, characterised by overtly smaller nuclei (arrows). (B) Staining abrogated in basal (small nuclei) cells (realised by DAPI staining, arrows) during out-competition with unlabelled 10^{-5} M kinin. Kinin-F, green; DAPI, blue. Scale bars = 50 μ m.

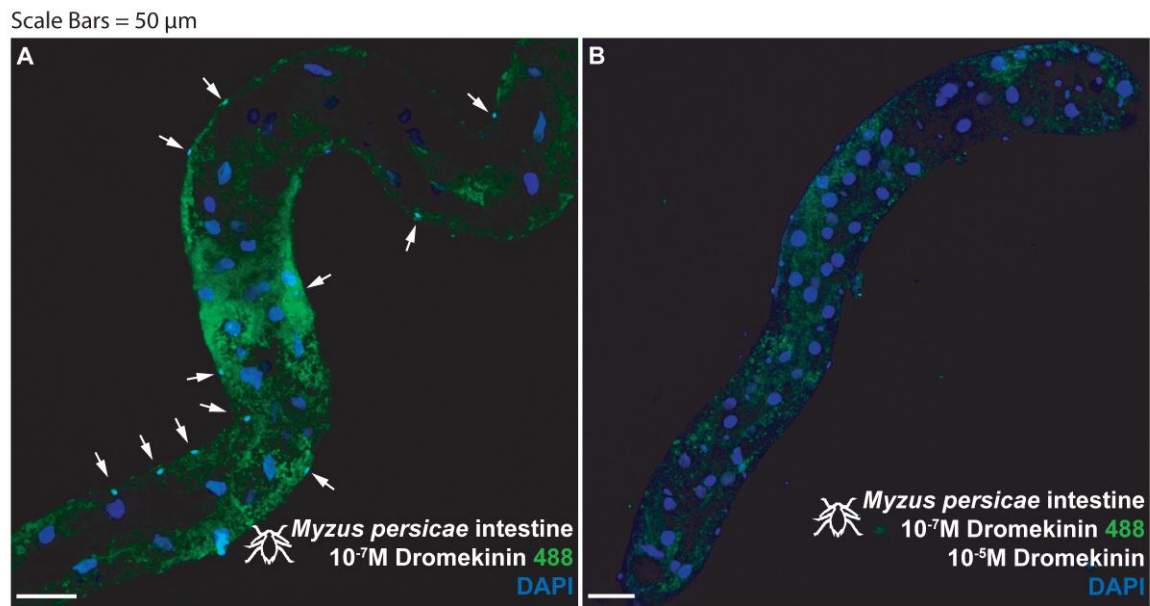


Figure 3 *Myzus persicae* stomach (midgut) stained with 10^{-7} M CAPA-1-F labelled with TMR C5-Maleimide. (A) Staining apparent at junctional area between the fore- and midgut (white box). (B) Higher magnification detail of staining associated with this junctional area. Staining is abrogated when outcompeted with unlabelled 10^{-5} M CAPA-1 (not shown). CAPA-1-F, red; DAPI, blue. Scale bars = 50 μ m.

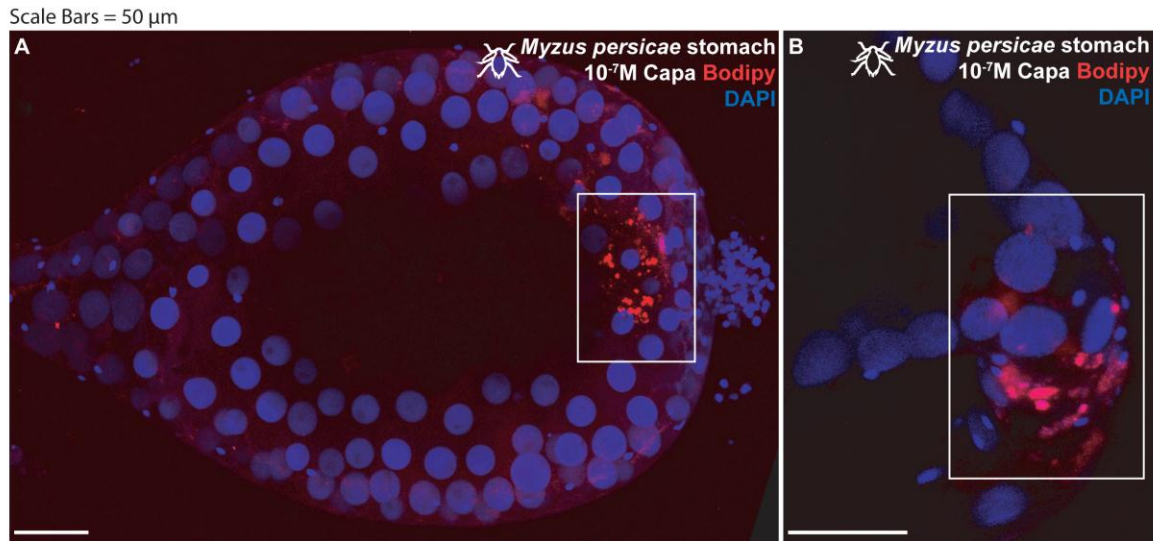


Figure 4 (A) Unstained *Myzus persicae* CNS, demonstrating baseline autofluorescent levels (488 nm excitation range; green). (B) cartoon schematic. (C) *Myzus persicae* brain and (D) VNC incubated with 10^{-7} M kinin-F labelled with alexafluor488. (C) Staining apparent in a bilateral ‘ladder’ of neurons and a set of more baso-lateral neurons in the suboesophageal ganglion (¹white box). Position of ¹white box indicated by ¹boxes in (A) and (B). Staining also apparent in symmetrical pairs of neurons/neuronal clusters in the ventro- to dorso-lateral protocerebrum (arrows). Some neurons obscured by cuticular material associated with the aphid feeding stylus (asterisk). (D) Little to no kinin-F staining apparent in the VNC, although a faint set of cells in the most distal tip of the abdominal ganglion (²white box) are consistently observed. Position of ²white box indicated by ²boxes in (A) and (B). (E) *Myzus persicae* brain and (F) VNC incubated with 10^{-7} M CAPA-1-F labelled with TMR C5-Maleimide. No apparent staining with CAPA-1-F in either brain or VNC. Kinin-F, green; CAPA-1-F, red; DAPI, blue. Scale bars = 50 μ m.

Scale Bars = 50 μ m

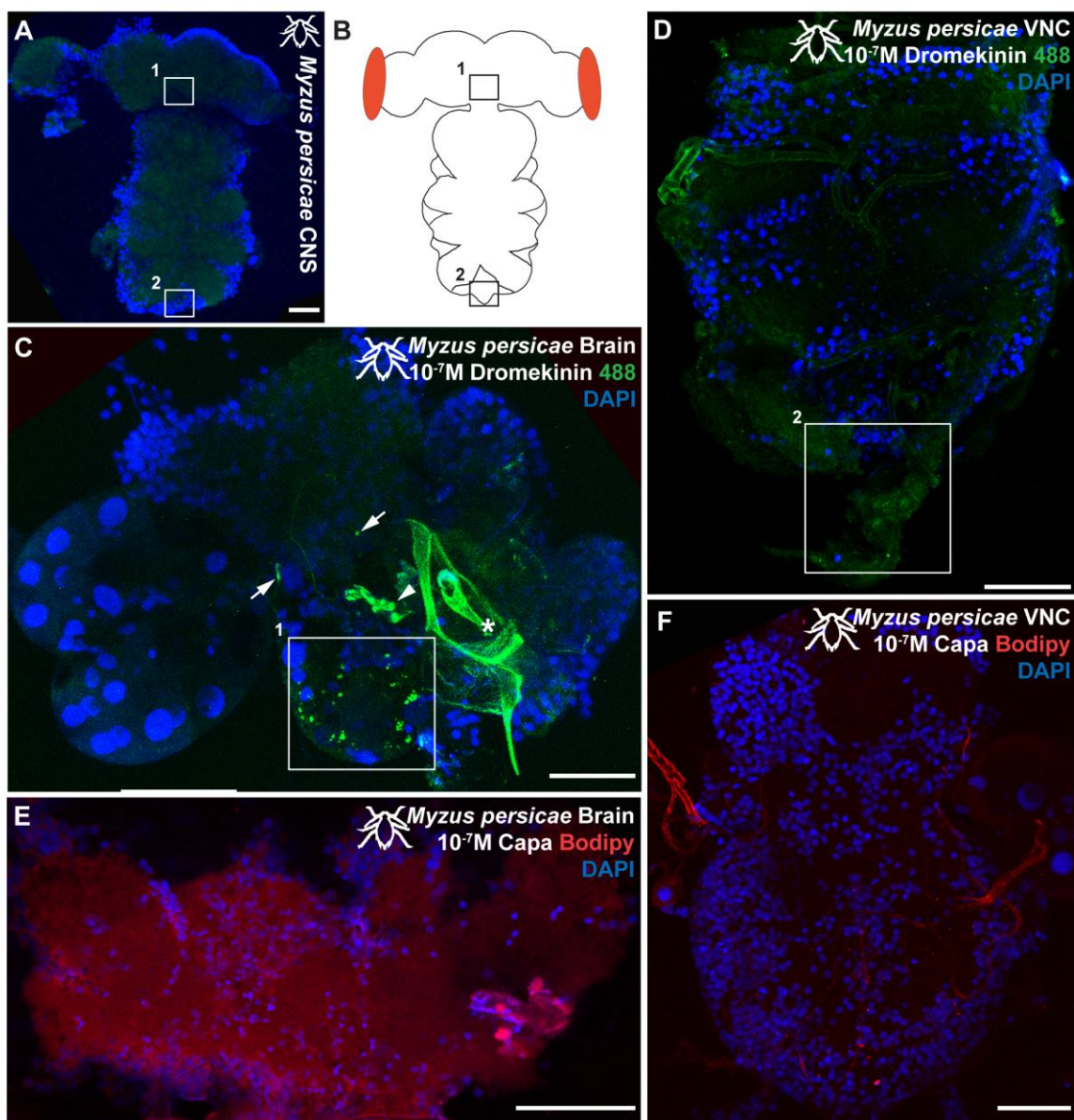


Figure 5 Effect of CAP2b and kinin analogue treatment on the survival of *Myzus persicae* (1) and *M. rosae* (2) under conditions of desiccation and starvation stress. Control aphids are indicated by the black line and analogue-treated aphids by the blue line. CAP2b analogues 1895 (a) and 2129 (b) were administered to a final concentration of $\times 10^{-5}$ M via microinjection and acted to significantly increase mortality relative to the control. CAP2b analogue 2125 (c) and kinin analogue 2139 (d) are presented to illustrate non-significant survival curves.

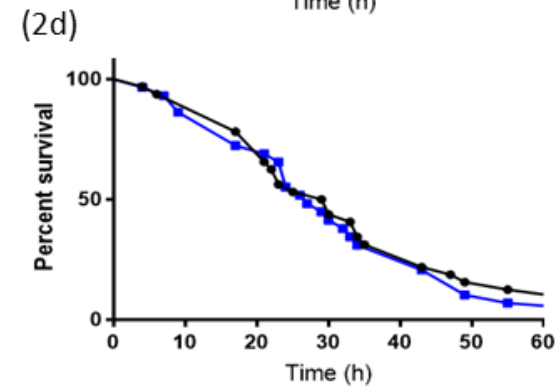
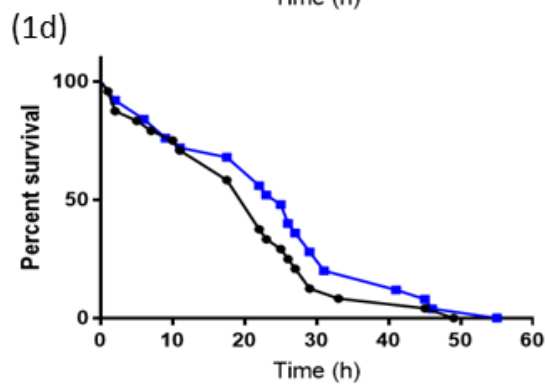
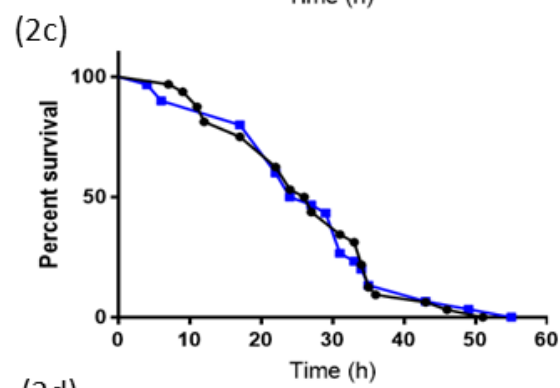
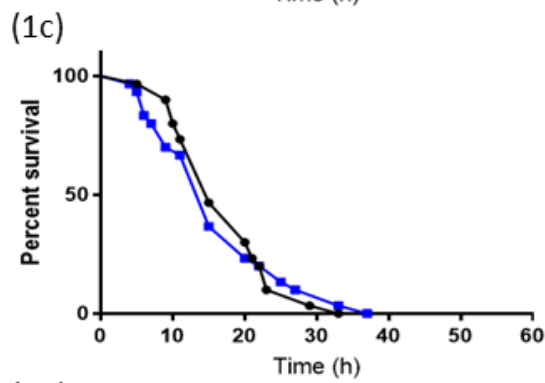
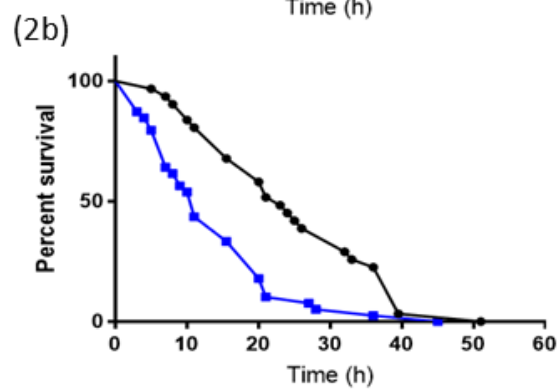
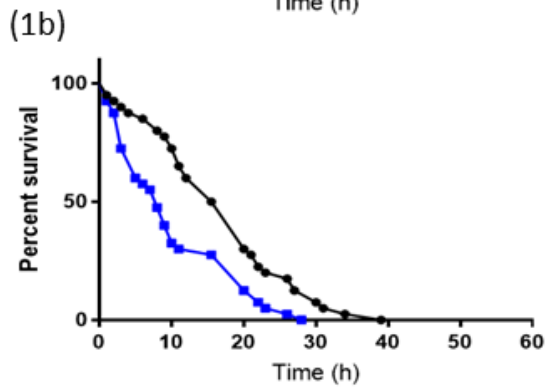
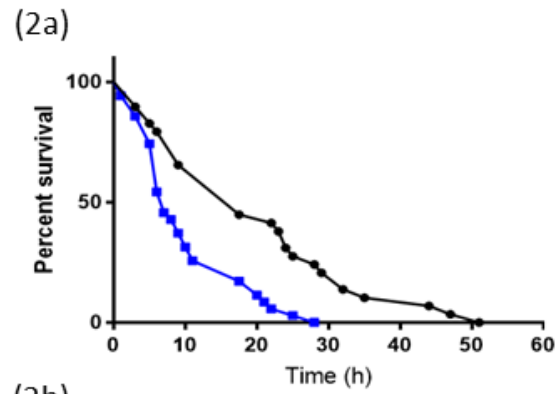
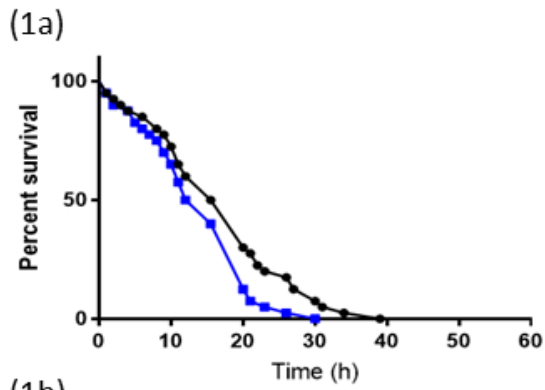


Figure 6 Survival curve calculated via Probit analysis of *Myzus persicae* pre-reproductive adults following a 1 hr exposure at the desired temperature. Raw data values are indicated by black circles.

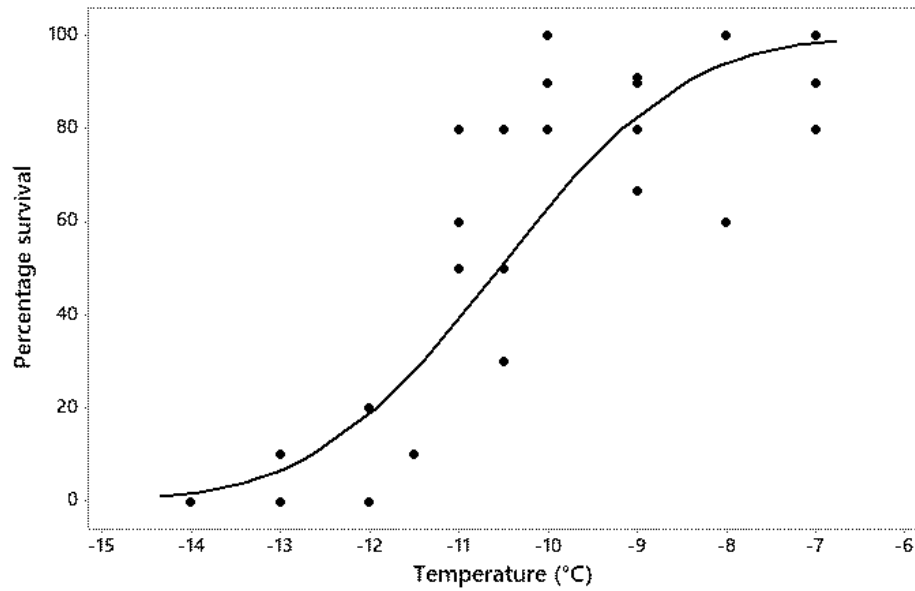


Figure 7 Mean \pm standard error proportion survival of *M. persicae* when treated with biostable peptide analogues (CAP2b/PK: 1895, 1896, 1902, 2089, 2123, 2125, 2129; kinin: 1728, 2139, 2139-Ac) via microinjection and subjected to a discriminating temperature for a 1 h exposure. Control groups are shown in black and peptide treatment groups in red.

