Structure-Activity Studies on the Hypertrehalosemic Hormone II of the Stick Insect Carausius morosus (Phasmatodea): Carbohydrate-Mobilization and Cardio-Stimulatory Activities

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The corpora cardiaca of the Indian stick insect, Carausius morosus, synthesize two decapeptide neuropeptides of the adipokinetic hormone (AKH) family, both of which can increase the trehalose levels in the hemolymph when the stick insect is ligated between the head and the thorax. Here, we use two biological assays to assess the potencies of 19 AKH analogs in ligated C. morosus: the carbohydrate-mobilizing assay measures the change in the levels of circulating carbohydrates following injection of a substance, while the semi-exposed heart assay measures a change in heart beat rate after the peptide is applied onto the heart. With the endogenous AKH (Carmo-HrTH-II) as lead peptide, we report here on seven naturally-occurring AKH peptides (bioanalogs) selected for testing because of a single or double amino acid replacement, or for being octapeptides. Single amino acid substitutions by an alanine residue at all positions of Carmo-HrTH-II, as well as analogs modified at the termini were also investigated to give a comprehensive view of ligand-receptor interaction at the physiological level in a hemimetabolous insect that practices thanatosis (feigning death). Only small changes are elicited in the bioassays, but the results from the two tests are comparable bar one or two anomalies. Results show that analogs modified at the termini have no or reduced activity. Regarding structural requirements of a ligand, the C. morosus AKH receptor appears to be strict: octapeptides are not preferred and many of the decapeptide analogs failed to reach 50% activity relative to Carmo-HrTH-II. The data implies that the AKH receptor in C. morosus mostly does not tolerate shorter peptides and single amino acid replacements in most places of the native AKH peptide. This information is important if environmentally friendly insect-specific pesticides are made based on an insect AKH as lead peptide: stick insects that are normally not viewed as pest insects may not be easily targeted by cross-reactive AKH mimetics directed at harmful insects, due to the very specific amino acid requirements to activate the C. morosus AKH receptor.

Keywords: adipokinetic hormone, hypertrehalosemic hormone, fuel mobilization, heart beat rate, Carausius morosus, Carmo-HrTH-II, structure-activity studies, stick insect
INTRODUCTION

Insects are not only well-known for their diversity and abundance but also for their influence on the biosphere and human life. The major anthropomorphic division in insect groups is made between those that are beneficial to mankind and those that are health risks or agricultural pests (Burn et al., 1987; Capinera, 2010; Gullan and Cranston, 2014). Increasingly, there is a strong interest in developing environmentally friendly insecticides that are selective and affect only the target (pest) species instead of all insects. Specifically, the development of hormone-like compounds that can be used in specific drug design to act as targeted pesticides are being considered (Altstein et al., 2000; Gäde and Goldsworthy, 2003; Audsley and Down, 2015). The compounds in mind are the insect’s neuropeptide hormones that control most of the key physiological processes such as development, reproduction, metabolism, behavior, muscle contraction including heart beat rate and diuresis.

A well-researched family of hormones are the adipokinetic hormones (AKHs) which are synthesized and released from the retrocerebral corpora cardiaca (CC); the AKHs are mainly tasked with mobilizing fuels (energy-rich metabolites) from fat body stores into the hemolymph and are identified as putative targets to develop new insecticides (see review by Marco and Gäde, 2020). The AKH peptide family is generally characterized by peptides having (a) a chain length of 8–10 amino acids; (b) post-translationally modified termini: a pGlu residue at the N-terminus and a carboxamide at the C-terminus; (c) either a Leu, Ile, Val or Phe residue at position 2; (d) a Thr or Asn residue at position 3; (e) an aromatic Phe or Tyr residue at position 4; (f) the branched amino acids Thr or Ser at position 5; (g) the aromatic residue Trp at position 8; (h) the simple amino acid Gly at position 9, and (i) variable amino acids at positions 6, 7, and 10 (see Gäde, 2009).

AKHs exert a biological effect via a G protein-coupled receptor (GPCR) and this system, the ligand-receptor pair, is the target for peptide mimetics to be developed (see reviews by Audsley and Down, 2015; Verlinden et al., 2015) in a fashion similar to the well-known beta blockers treating hypertension in human medicine. Two standard methods have been used to investigate AKH ligand-receptor interactions in structure-activity relationship (SAR) studies; the oldest being an indirect in vivo biological assay in which ligands are tested in live animals, and the result of a signal transduction cascade is measured, e.g., the release of lipids/carbohydrates into the hemolymph, or the activation of glycogen phosphorylase. This has been done for AKH bioanalogues and synthetic analogs in locusts (see, for example, Stone et al., 1978; Gäde, 1990, 1993; Poulos et al., 1994; Goldsworthy et al., 1997), lepidopterans (Fox and Reynolds, 1991; Ziegler et al., 1991, 1998; Marco and Gäde, 2015, 2019) and cockroaches (Gäde, 1986, 1990, 1992; Ford et al., 1988; Hayes and Keeley, 1990; Gäde and Hayes, 1995). The second and more recent method of conducting SARs is via a direct in vitro receptor assay; the prerequisite is to have knowledge of the AKH receptor sequence: this is in general (with a few exceptions) only the case for those insects where the whole genome is known. To date, detailed receptor assay SAR studies with bioanalogues and specifically modified peptides have only been performed with dipteran species, Drosophila melanogaster, Anopheles gambiae and Glossina morsitans (Caers et al., 2012, 2016). In general, the results of these in vitro assays agree with those generated by in vivo biological assays, i.e., that the conserved aromatic amino acids at position 4 and 8 are important for receptor-peptide interactions, as well as (in the majority of cases) the blocked termini, whereas amino acids at positions 7 and 10 are not always that crucial. It also appears to emerge that the receptor of those species that have two or more endogenous AKHs, such as Periplaneta americana, Locusta migratoria, and Hippotion eson, seem to tolerate a wider variety of ligand modifications than an insect with a single endogenous AKH, such as Blaberus discoidalis and Aedes aegypti (see references above; Marchal et al., 2018; Wahedi et al., 2019).

AKHs are also known for their myotropic effects, especially to increase the rate of heart beat (Chowański et al., 2016); SAR studies have only been done in the cockroach P. americana to a certain extent (Baumann et al., 1990), and a few bioanalogues were tested on the heart of the stick insect Baculum extradentatum (Malik et al., 2012).

In the current study, a member of the order Phasmatodea is investigated with respect to metabolic (hypertrehalosemic) and myotropic (cardio-stimulatory) activity of AKH. Insects of this order are well known to be kept as pets, although there are also reports on the pest status of certain phasmid species, especially in private gardens on ornamental plants in the United States of America (Griffiths and Picker, 2011). The subject of the current study, the Indian stick insect Carausius morosus, is already well-known in laboratory research of neuropeptide hormones (see for example Miksys et al., 1997; Predel and Gäde, 1999; Lorenz et al., 2000; Liessem et al., 2018), and has also become a model organism for neurobiology, especially studying the control of locomotion (see for example, Bidaye et al., 2018). With regards to AKH research on C. morosus, two near-identical decapetide members of the AKH family were isolated from the corpora cardiaca, and these peptides had no effect on hemolymph metabolite levels in conspecific assays (Gäde, 1979). Three years later, a hypertrehalosemic effect of conspecific CC extract was demonstrated in adult C. morosus only when a ligature was applied to the insect (between the head and the first pair of legs) before injection of the CC extract (Gäde and Lohr, 1982). The exploratory study of 1982 further revealed that the increase in circulating trehalose was relatively small (about 4–7 mg ml⁻¹); the highest response was recorded in ligated 6th instar larvae shortly before the final molt; and there was evidence of ligand preference, i.e., the AKH receptor of the stick insect recognized the conspecific AKHs but not that of lepidopteran species, the migratory locust or of the decapod crustaceans (Gäde and Lohr, 1982). The two stick insect AKHs were sequenced by fast atom bombardment mass spectrometry (FABMS) (Gäde, 1985; Gäde and Rinehart, 1987): the later eluting 2nd peak contained most of the peptidic material and has the code-name C. morosus HRTH-II; the earlier eluting peak (code-name C. morosus HRTH-I) was shown to be identical in the primary sequence to Carmo-HRTH-II but a hexose is bound in an unorthodox manner to the Trp residue at position 8 (Gäde et al., 1992).
Through the heroic collection of 2000 CC and the use of a sensitive 800 MHz nuclear magnetic resonance spectrometer equipped with a cryoprobe, it was possible to show unequivocally that an α-mannopyranose is bound to Trp8 of Carmo-HrTH-I in an unusual C-glycosylated fashion (Munte et al., 2008). Recently, a transcriptomic and neuropeptidomic analysis of the central nervous system of C. morosus revealed only one AKH precursor although both Carmo-HrTH-I and -II were confirmed in mass spectrometry of tissues (Liessem et al., 2018), thus, the one peptide precursor is modified post-translationally to form both versions of C. morosus AKHs.

Recently it was also shown that both AKH peptides of C. morosus are capable of increasing the rate of heart contraction in ligated stick insects: the application of synthetic Carmo-HrTH-II in doses above $6.67 \times 10^{-8}$ M increased the heart beat rate significantly and maximally (i.e., higher peptide doses had no further increase on the heart beat rate; Marco et al., 2018). The frequency of heart contractions in C. morosus at rest was found to be much lower than that recorded in more active insect species and again, the endogenous AKH peptide could not affect heart contractions in non-ligated C. morosus while small increases in the heart beat rate were recorded in ligated stick insects (Marco et al., 2018). Prior to this study, an influence of AKH on stimulating the heart frequency of the Vietnamese stick insect, B. extradentatum, was also shown in decapitated specimens (Malik et al., 2012). The low heart rate and the small increases in metabolic reactions to endogenous AKHs are interpreted as evolutionary consequences of the cryptic defenses of stick insects where they shut down the metabolism to play dead (thanatosis) to avoid detection from predators, instead of entering into the fight or flight mode (Marco et al., 2018).

Based on the above-mentioned results, the present study was initiated with the following aim: to understand in detail the requirements of the AKH receptor with respect to structural features of the ligand. Although a number of detailed SAR studies have been performed on the AKH system in insects (references see above), there is only one case according to our information where an insect that has one decapeptide as AKH ligand has been tested; this is the cockroach B. discoidalis and its AKH Bladi-HrTH (Ford et al., 1988; Hayes and Keeley, 1990). That study and the companion research on P. americana (Gäde and Hayes, 1995), an insect with AKH octapeptides, show that the receptors have some different properties. Since the AKH receptor of C. morosus was not structurally known at the time of the current study, we performed in vivo bioassays and tested for a metabolic response (hypertrehalosemic activity) and a myotropic response (heart beat rate). In this way we also hoped to establish whether the putative receptor in the fat body tissue and in the heart muscle tissue are very likely identical. Bioanalogues and specifically altered peptides were chosen/designed to obtain the following information on structural requirements for interaction with the C. morosus AKH receptor: (a) acceptable chain length of the ligand, (b) the importance of the AKH termini, and (c) the side chain of each amino acid in the decapeptide.

## MATERIALS AND METHODS

### Insects

Indian stick insects (Carausius morosus) were reared under crowded conditions at approx. 25 ± 2°C, 65% RH and a 12 h light: 12 h dark regime. The stick insect nymphs were fed with fresh ivy (Hedera helix) leaves supplied twice a week, while the adults were provided with fresh twigs of mirror bush (Coprosma repens) about twice a week.

### Peptides and Corpora Cardiaca Extract

For sequence information of the various synthetic peptides (see Tables 1–3).

The endogenous hypertrehalosemic hormone Carmo-HrTH-I was isolated from the CC of C. morosus as previously described by Gäde (1985). Synthetic Carmo-HrTH-II was purchased from

### TABLE 1 | The biological effect (metabolic) of the termini of Carmo-HrTH-II in ligated 6th instar Indian stick insects (Carausius morosus).

| Treatment (10 µl injected) and amino acid sequencea (20 pmol peptide injected) | Hemolymph carbohydrates (mg ml⁻¹) | % Activity relative to the effect of Carmo-HrTH-II |
|---|---|---|
| | n | 0 min | 90 min | Difference | Pα |
| Distilled water | 13 | 8.1 ± 3.1 | 8.5 ± 2.5 | 0.4 ± 1.5 | NS |
| Carmo-HrTH-II | | | | | |
| pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | 10 | 9.4 ± 1.5 | 14.9 ± 1.9 | 5.4 ± 1.1 | 0.00001 |
| pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | 12 | 8.0 ± 1.6 | 12.9 ± 1.9 | 4.9 ± 1.1 | 0.00001 |
| [N-Ac]Ala-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | 10 | 9.0 ± 1.3 | 10.6 ± 2.0 | 1.5 ± 1.1 | 0.002 |
| Carmo-HrTH-II: Nonapeptide, free N-terminus, Leu1 | | | | | |
| [N-Ac]Ala-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | 14 | 8.8 ± 1.7 | 9.5 ± 1.5 | 0.7 ± 1.3 | NS |
| Carmo-HrTH-II: free C-terminus | | | | | |
| pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | 21 | 8.8 ± 2.1 | 10.4 ± 2.6 | 1.6 ± 1.6 | 0.0002 |

The data are presented as Mean ± S.D. aAmino acid substitutions in Carmo-HrTH-II analogs are highlighted. bTryptophan residue is mannosylated. cNot significantly different from the effect of Carmo-HrTH-II (which was set as 100%), as determined by ANOVA and post hoc Scheffe’s test. dPaired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.
TABLE 2 | The biological effect (metabolic) of selected AKH bioanalogs in ligated 6th instar Indian stick insects (*Carausius morosus*).

| Treatment (10 µl injected) and amino acid sequencea (20 pmol peptide injected) | Hemolymph carbohydrates (mg ml⁻¹) | % Activity relative to the effect of Carmo-HrTH-II |
|---|---|---|
| | | n | 0 min | 90 min | Difference | P# |
| Decapeptides | | Distilled water | 13 | 8.1 ± 3.1 | 8.5 ± 2.5 | 0.4 ± 1.5 | NS |
| | | Carmo-HrTH-II | 10 | 9.4 ± 1.5 | 14.9 ± 1.9 | 5.4 ± 1.1 | 0.00001 |
| | | Phyle-CC | 10 | 9.4 ± 1.5 | 14.9 ± 1.9 | 5.4 ± 1.1 | 0.00001 |
| | | Locmi-AKH-I | 12 | 8.9 ± 1.3 | 9.9 ± 1.2 | 1.0 ± 1.2 | 0.002 |
| | | Phymo-AKH | 10 | 9.6 ± 1.8 | 10.5 ± 1.4 | 0.9 ± 1.7 | NS |
| | | Rommi-CC | 10 | 8.0 ± 1.4 | 10.6 ± 1.6 | 2.6 ± 1.5 | 0.0004 |
| Octapeptides | | Peram-CAH-II | 18 | 9.3 ± 1.8 | 11.0 ± 1.1 | 1.7 ± 2.0 | 0.002 |
| | | Aedae-AKH | 10 | 8.1 ± 1.4 | 8.8 ± 1.2 | 0.7 ± 1.1 | NS |
| | | Pyrap-AKH | 10 | 10.3 ± 2.4 | 11.1 ± 1.8 | 0.8 ± 1.6 | NS |
| | | Locmi-AKH-I | 12 | 8.9 ± 1.3 | 9.9 ± 1.2 | 1.0 ± 1.2 | 0.002 |
| | | Phymo-AKH | 10 | 9.6 ± 1.8 | 10.5 ± 1.4 | 0.9 ± 1.7 | NS |
| | | Rommi-CC | 10 | 8.0 ± 1.4 | 10.6 ± 1.6 | 2.6 ± 1.5 | 0.0004 |

**a**Amino acid substitutions in Carmo-HrTH-II analogs are highlighted. **b**Not significantly different from the effect of Carmo-HrTH-II (which was set as 100%), as determined by ANOVA and post hoc Scheffe’s test. The data are presented as Mean ± S.D. **#**Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.

TABLE 3 | The biological effect (metabolic) of single amino acid substitutions in Carmo-HrTH-II in ligated 6th instar Indian stick insects (*Carausius morosus*).

| Treatment (10 µl injected) and amino acid sequencea (20 pmol peptide injected) | Hemolymph carbohydrates (mg ml⁻¹) | % Activity relative to the effect of Carmo-HrTH-II |
|---|---|---|
| | | n | 0 min | 90 min | Difference | P* |
| Distilled water | | 13 | 8.1 ± 3.1 | 8.5 ± 2.5 | 0.4 ± 1.5 | NS |
| Carmo-HrTH-II | | 10 | 9.4 ± 1.5 | 14.9 ± 1.9 | 5.4 ± 1.1 | 0.00001 |
| pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | | 10 | 9.4 ± 1.5 | 14.9 ± 1.9 | 5.4 ± 1.1 | 0.00001 |
| Ala2-Carmo-HrTH-II | | 10 | 9.4 ± 1.5 | 14.9 ± 1.9 | 5.4 ± 1.1 | 0.00001 |
| pGlu-Leu-Thr-Phe-Pro-Asn-Trp-Gly-Thr amide | | 10 | 11.4 ± 2.3 | 16.8 ± 1.7 | 5.4 ± 1.7 | 0.00003 |
| Ala3-Carmo-HrTH-II | | 10 | 11.4 ± 2.3 | 16.8 ± 1.7 | 5.4 ± 1.7 | 0.00003 |
| pGlu-Leu-Thr-Phe-Pro-Asn-Trp-Gly-Thr amide | | 10 | 10.3 ± 2.4 | 11.1 ± 1.8 | 0.8 ± 1.6 | NS |
| Ala5-Carmo-HrTH-II | | 20 | 8.6 ± 2.2 | 9.1 ± 2.4 | 0.5 ± 2.3 | NS |
| pGlu-Leu-Thr-Pro-Asn-Trp-Gly-Thr amide | | 10 | 10.2 ± 1.2 | 10.1 ± 1.3 | 0.1 ± 0.1 | NS |
| Ala6-Carmo-HrTH-II | | 10 | 10.2 ± 1.2 | 10.1 ± 1.3 | 0.1 ± 0.1 | NS |
| pGlu-Leu-Thr-Phe-Pro-Asn-Trp-Gly-Thr amide | | 10 | 9.5 ± 1.2 | 11.1 ± 0.9 | 1.6 ± 1.1 | 0.001 |
| Ala7-Carmo-HrTH-II | | 10 | 11.3 ± 2.0 | 12.0 ± 2.2 | 0.7 ± 0.6 | 0.003 |
| pGlu-Leu-Thr-Phe-Pro-Asn-Trp-Gly-Thr amide | | 10 | 8.5 ± 2.1 | 8.3 ± 1.9 | 0.1 ± 1.3 | NS |
| Ala9-Carmo-HrTH-II | | 10 | 8.5 ± 2.1 | 8.3 ± 1.9 | 0.1 ± 1.3 | NS |
| pGlu-Leu-Thr-Phe-Pro-Asn-Trp-Gly-Thr amide | | 10 | 9.5 ± 1.9 | 11.0 ± 1.5 | 1.5 ± 0.9 | 0.0004 |
| Ala10-Carmo-HrTH-II | | 10 | 9.5 ± 1.9 | 11.0 ± 1.5 | 1.5 ± 0.9 | 0.0004 |
| pGlu-Leu-Thr-Phe-Pro-Asn-Trp-Gly-Proamide | | 10 | 9.9 ± 2.3 | 12.4 ± 1.6 | 2.5 ± 1.0 | 0.00002 |

**a**Amino acid substitutions in Carmo-HrTH-II analogs are highlighted. Data presented as Mean ± S.D. *****Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.
Peninsula Laboratories (Belmont, CA, United States) together with Locmi-AKH-I and Peram-CAH-I. Phyle-CC and Pyrap-AKH were synthesized by Dr. R. Kellner (Merck, Germany). Aedae-AKH was made by Genscript Corporation (United States). AKH were synthesized by Dr. S. Kyin (Biotechnology Centre, University of Illinois, Urbana-Champaign, United States). Analogos of Carmo-HrTH-II with single amino acids replaced by Ala were purchased from Pepmic Co., Ltd (Suzhou, China). Stock solutions were made by dissolving 1 mg of each peptide in 1 ml min−1 using distilled water. The various peptide solutions were subsequently monitored for purity and quantified via reversed phase-high performance liquid chromatography (Gilson RP-HPLC system) with fluorescence detection (276 nm excitation, 350 nm emission; Gäde, 1985) and with a gradient of 43–53% solvent B in 20 min at a flow rate of 1 ml min−1 (Nucleosil C18 column; Solvent A: 0.11% TFA in water; solvent B: 60% acetonitrile with 0.10% TFA).

Corpora cardiaca (with the corpora allata attached) were dissected from the head of adult Carausius morosus into 80% methanol, the cell contents were extracted on ice via sonication (Branson sonifier cell disruptor), followed by centrifugation and the resulting supernatant was dried in a vacuum concentrator. The dried corpora cardiaca extracts were then reconstituted in distilled water for use in carbohydrate-mobilization assays.

Bioassays

Carbohydrate-Mobilization Assay

Sixth instar nymphs that were 1–2 days before molting were neck-ligated and used in the hypertrehalosemic in vivo assay as described in detail by Gäde and Lohr (1982). Stock peptide solutions (5 pmol µl−1) were diluted with distilled water to the desired concentration in 10 µl, which was injected into the stick insect nymphs.

Semi-Exposed Heart Bioassay

The semi-exposed heart bioassay was carried out as described (Marco et al., 2018) with adult stick insects between 1 and 2 months old that were ligated at the neck. Briefly, a ventral incision of the abdomen exposed the ventral cavity with the internal organs; a few drops of stick insect saline (pH 6.6; 15 mM NaCl, 18 mM KCl, 7.5 mM CaCl₂, 2 mM HEPES, 50 mM MgCl₂, and 184 mM glucose according to Ejaz and Lange, 2008) were added to the exposed part. The semi-isolated heart preparation was viewed with a dissecting microscope (12-fold magnification), and the number of observed heart beats (contractions of the heart muscle) was counted during a fixed period using a manual tally counter and a timer. Once the heart had stabilized, saline was replaced with the test solution. For this the peptide stock solution of 5 pmol µl−1 was diluted with stick insect saline to the desired concentration and a final volume of 150 µl was used for each test.

Heart rates under normal saline served as controls. The change in heart rate after the application of the test solution was calculated as an average of the first four counts and was compared to the average heart rates counted in saline before the application of the test solution. This comparison of change in heart rates was done for all the peptides tested.

Statistical Analyses

Student’s paired t-test was used to compare the concentrations of carbohydrates in the hemolymph, as well as the heart rates of Carausius morosus before and after the subjection to the test solution. One-way analysis of variance (ANOVA) followed by Scheffe’s multiple comparison test was used to compare the hypertrehalosemic and cardio-stimulatory effects among Carausius morosus tested with different peptides. Differences were considered significant at p < 0.05 for all the tests.

RESULTS

Hypertrehalosemic Effects of the Endogenous Peptides

In the first series of experiments, various doses of synthetic Carmo-HrTH-II and corpora cardiaca (CC) extract of Carausius morosus were used to determine the maximal hypertrehalosemic response of Carausius morosus. Hemolymph samples were taken from 17 to 18 days old 6th instar nymphs 2 h after they were neck-ligated and then 90 min after either CC extract or synthetic Carmo-HrTH-II was injected. Ligated nymphs injected with distilled water served as controls for handling stress. As depicted in Figures 1A,B, various doses of both Carmo-HrTH-II and CC extract increased the level of carbohydrates in the hemolymph.
in a dose-dependent manner. A dose of 0.04 pmol of synthetic Carmo-HrTH-II and the equivalent of 0.005 pairs of corpora cardiaca (pCC) were sufficient to give significant increases in hemolymph carbohydrates ($p < 0.05$). With synthetic Carmo-HrTH-II, doses of 7.5–60 pmol gave an average increase of 5.2 ± 0.4 mg ml$^{-1}$ and the post hoc Scheffe's test revealed that there was no significant difference between the effect of these doses ($p > 0.05$). Doses of 0.1–0.5 pCC gave an average increase of 5.6 ± 0.6 mg carbohydrates ml$^{-1}$ hemolymph (Figure 1B); again, statistical analyses reveal no significant difference between the effect of these doses ($p > 0.05$). An anomalous result was obtained after injection with 0.05 pCC extract: a much higher increase in carbohydrate concentration (8.5 ± 1.0 mg ml$^{-1}$) was measured than from any other dose tested (Figure 1B), and this increase differed significantly ($p < 0.05$) from all the higher CC extract doses; moreover, such an increase was not achieved by any injection of synthetic Carmo-HrTH-II even at high doses (see Figure 1A).

Based on these pilot experiments, we selected a dose of 20 pmol for testing the analogs of Carmo-HrTH-II since such a dose of our lead compound, Carmo-HrTH-II had achieved maximal hypertrehalosemic activity.

Hypertrehalosemic Response of Synthetic Analogs of Carmo-HrTH-II

The Biological Effect of the Termini of Carmo-HrTH-II in C. morosus

AKH peptides are characterized by a pGlu in position 1 and an amidated C-terminus. This is believed to be an effective block against exopeptidases in the hemolymph of the insect, resulting in a longer half-life of the peptide (Oudejans et al., 1996). Carmo-HrTH-II analogs were designed to specifically explore how carbohydrate mobilization is affected by changes to the N terminal amino acid of the decapeptide. The results are shown in Table 1. When the termini of Carmo-HrTH were modified (i.e., N-terminal pGlu replaced with an acetylated Ala residue, or a free acid at the C-terminus instead of an amidation) the hypertrehalosemic activity was severely reduced from 100% to a mere 30%, while a nonapeptide lacking a pGlu and having, thus, a Leu residue in position 1 had no significant biological effect.

The Biological Effect of AKH Bioanalogs in C. morosus

Functional AKHs are either composed of eight, nine, or 10 amino acids; C. morosus synthesizes only decapeptide AKHs. The biological effect of shorter chain lengths (octapeptides) were therefore investigated, along with single or double amino acid substitutions in naturally occurring decapeptide AKHs to ascertain the flexibility of the C. morosus receptor that usually sees and responds only to two decapeptides of the same amino acid sequence.

Peram-CAH-II has the same 8 amino acids as in the endogenous decapeptide of the stick insect, Carmo-HrTH; this is, however, only sufficient to elicit a 30% hypertrehalosemic response (Table 2). The remaining octapeptides tested with single substitutions at position 3 and 7 had no significant activity.

Of the decapeptide bioanalogs, only Phyle-CC (Ser$^2$-Asn$^3$ instead of Leu$^2$-Thr$^3$) increased the hemolymph carbohydrates as high as Carmo-HrTH-II ($p > 0.05$), while Rommi-CC (Val$^2$-Asn$^3$ instead of Leu$^2$-Thr$^3$) elicited a response of 48% of the maximal possible hypertrehalosemic effect. The remaining analogs were virtually unable to increase the hemolymph carbohydrates (Table 2).

The Biological Effect of Single Amino Acid Replacements in Carmo-HrTH-II

The relative importance of each amino acid of Carmo-HrTH-II was investigated via a series of analogs in which one amino acid was substituted with an alanine residue (Table 3). In this way we can make inferences about the relative importance of the side chains of the original residues in activating the stick insect AKH receptor. The substitution of an aromatic amino acid residue (i.e., Phe$^4$ or Trp$^8$) with Ala eliminated AKH activity (Table 3). An Ala replacement of Thr in position 3 and 5 also gave no hypertrehalosemic effect, while a very small increase in carbohydrates was observed after injection of an Ala$^8$ analog (thus, replacing Asn$^7$). Ala$^8$ and Ala$^9$ replacements of Pro and Gly, respectively (Table 3) resulted in a marked reduction of biological activity (around 30%), whereas Ala$^{10}$. Carmo-HrTH-II (in place of Thr$^{10}$) resulted in about half of the maximal possible hypertrehalosemic effect. In contrast, Ala$^8$-Carmo-HrTH-II increased the hemolymph carbohydrates to the same extent as did Carmo-HrTH-II (Table 3).

Effect of Endogenous Neuropeptides and Synthetic Analogs of Carmo-HrTH-II on the Heart Rate of C. morosus

Adult Indian stick insects without a neck ligature are unable to respond with an increased heart beat rate upon the application of various doses of Carmo-HrTH-II (see Marco et al., 2018). In neck-ligated stick insects, however, the application of 20 pmol of the endogenous AKH peptides (Carmo-HrTH-I and -II) on the heart preparation increases the heart rate significantly from 41 beats min$^{-1}$ to 53 beats min$^{-1}$ ($p < 0.05$; Table 4). The modified Carmo-HrTH-II with the acetylated Ala residue at the N-terminus did not significantly alter the rate of heart beat, whereas the heart rate increased (58% of the maximal response possible) after the application of the free acid-Carmo-HrTH-II and the Leu$^1$ nonapeptide (Table 4). The post hoc test revealed that there was no significant difference between the potencies of Carmo-HrTH-I and -II ($p > 0.05$) but there was a significant difference between the result of Carmo-HrTH-II and those of the terminal modified analogs (Table 4).

Of the bioanalogs tested, the increase in heart rate caused by the decapeptides Rommi-CC and Phyle-CC was highly significant and did not differ significantly from that caused by Carmo-HrTH-II ($p > 0.05$).

None of the systematically altered Ala analogs of Carmo-HrTH-II and none of the octapeptidic bioanalogs tested (Tables 5, 6) increased the heart rate significantly, except seemingly Ala$^3$-Carmo-HrTH-II (Table 6). However, one-way ANOVA followed by the post hoc Scheffe's test revealed that the response to Ala$^3$-Carmo-HrTH-II is not significantly different from those of the rest of the Ala-analogs nor does it differ from the saline effect ($p > 0.05$). The data from all the systematically
altered analogs and saline differed significantly from that of Carmo-HrTH-II (p < 0.0005).

DISCUSSION

The peptides of the AKH/RPCH family are mainly known for their involvement in the regulation of energy metabolism, specifically the mobilization of stored fuel metabolites (lipid, carbohydrates or proline), although the AKHs are pleiotropic (Gäde, 1997; Marco and Gäde, 2020). In the stick insect, Carausius morosus, the endogenous members of the AKH/RPCH family act as hypertrehalosemic hormones (HrTHs) and have a stimulatory effect on heart contraction under certain conditions. The present study, thus, aimed to investigate the importance of structural features of the native HrTHs necessary to interact with the receptor on the fat body and on dorsal vessel cells of C. morosus to trigger a response that culminate in the release of carbohydrates into the hemolymph, and an increase in heart rate. Additionally, we were interested in whether the receptor features

### TABLE 4 | The biological effect (myotropic) of the termini of Carmo-HrTH-II in ligated 6th instar Indian stick insects (Carausius morosus).

| Treatment (150 µl applied) and amino acid sequencea (20 pmol peptide) | Heart rate (beats min−1) | % Activity relative to the effect of Carmo-HrTH-II |
|---|---|---|
| | n | Pre-application | Post-application | Difference | P# |
| Saline | 12 | 40 ± 4 | 41 ± 1 | 1 ± 2 | NS |
| Carmo-HrTH-II | 8 | 41 ± 4 | 53 ± 5 | 12 ± 5 | 0.0002 | 100 |
| pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | 8 | 41 ± 2 | 54 ± 2 | 13 ± 2 | 0.002 | 108b |
| Carmo-HrTH-II: [N-Ac]Ala | 8 | 39 ± 6 | 39 ± 6 | 0 ± 0 | NS |
| [N-Ac]Ala-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | 9 | 39 ± 6 | 46 ± 5 | 7 ± 2 | 0.0001 | 58 |
| Carmo-HrTH-II: free C-terminus | 7 | 40 ± 5 | 47 ± 3 | 7 ± 3 | 0.0008 | 58 |

*a Amino acid substitutions in Carmo-HrTH-II analogs are highlighted. * Tryptophan residue is mannosylated. b Not significantly different from the effect of Carmo-HrTH-II (which was set as 100%), as determined by ANOVA and post hoc Scheffe’s test. The data are presented as Mean ± S.D. # Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.

### TABLE 5 | The biological effect (myotropic) of selected AKH bioanalogs in ligated 6th instar Indian stick insects (Carausius morosus).

| Treatment (150 µl applied) and amino acid sequencea (20 pmol peptide) | Heart rate (beats min−1) | % Activity relative to the effect of Carmo-HrTH-II |
|---|---|---|
| | n | Pre-application | Post-application | Difference | P# |
| Saline | 12 | 40 ± 4 | 41 ± 1 | 1 ± 2 | NS |
| Decapeptides | | | | | |
| Carmo-HrTH-II | | | | | |
| pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-Gly-Thr amide | 8 | 41 ± 4 | 53 ± 5 | 12 ± 5 | 0.0002 | 100 |
| Phyle-CC | 5 | 38 ± 7 | 46 ± 6 | 8 ± 2 | 0.0005 | 67b |
| Locmi-AKH | 4 | 40 ± 4 | 43 ± 4 | 3 ± 1 | 0.002 | 25 |
| Octapeptides | | | | | |
| Aedae-AKH | 6 | 37 ± 3 | 38 ± 4 | 1 ± 1 | 0.04 | 8 |
| Pyrap-AKH | 6 | 39 ± 3 | 57 ± 4 | 18 ± 6 | 0.0007 | 150b |

*a Amino acid substitutions in Carmo-HrTH-II analogs are highlighted. b Not significantly different from the effect of Carmo-HrTH-II (which was set as 100%), as determined by ANOVA and post hoc Scheffe’s test. The data are presented as Mean ± S.D. # Paired t-test was used to calculate the significance between pre-and post-injection values. NS, not significant.
of *C. morosus* would more or less mirror those of the cockroach *B. discoidalis* – the only insect with a sole decapeptide AKH that had been investigated via SAR studies to date.

**Hypertrehalosemic Response in *C. morosus***

Pioneering metabolic studies with *C. morosus* (Gäde and Lohr, 1982) had revealed that the concentration of total hemolymph carbohydrates and the amount of fat body glycogen at the end of the 6th instar stage were usually higher than in adults, and that ligated stick insects show a clear and consistent hypertrehalosemic response when about to molt into adults (i.e., late-6th instar stage). The early study also demonstrated unequivocally that the ligature itself had no impact on the concentration of carbohydrates in the body over the measured period (Gäde and Lohr, 1982). The present study, thus, used carefully staged 6th instar *C. morosus* specimens to confirm that, indeed, a maximal hypertrehalosemic response was attained upon injection of a crude extract of conspecific corpora cardiaca, or the endogenous AKH members, Carmo-HrTH-I and -II. Once this was ascertained, we performed structure-activity response (SAR) studies to investigate how specific changes to the primary amino acid sequence, we predict that the AKH receptor in this insect species may show a low tolerance for accommodating differences in peptide chain length (e.g., octapeptides and nonapeptides), as well as for particular amino acid substitutions in the peptide chain. The opposite was shown to be the case in the moth *Hipppotion eson* that produces and reacts positively to five endogenous AKHs of varying chain length and sequence (Marco and Gäde, 2015, 2019).

One of the characteristic features of AKH peptides are their blocked termini: a pyroglutamic acid (pGlu) in position 1, and an amidated C-terminus, which renders protection to the ligand from exopeptidases in the hemolymph of the insect, and therefore results in a longer half-life of the peptide to achieve its hormonal effect. The current study confirms the importance of the N-terminal pGlu and C-terminal amide of Carmo-HrTH-II, the lead peptide: the terminally modified analogs showed only a slight hypertrehalosemic activity and none at all, when the N-terminal pGlu residue was eliminated and, hence, an unprotected nonapeptide was tested. Previous *in vivo* receptor binding assays had also reported reduced or no activity when one of the blocking termini residues was removed or the pGlu was substituted by other blocked amino acids (Gäde, 1990; Ziegler et al., 1991, 1998; Gäde and Hayes, 1995; Lee et al., 1997; Marco and Gäde, 2015). In addition, *in vitro* binding assays on the flies would more or less mirror those of the cockroach *B. discoidalis* – the only insect with a sole decapeptide AKH that had been investigated via SAR studies to date.
D. melanogaster and G. morstians reported a decline in activation of the expressed AKH receptor when N-terminal acetylata- 
Ala analogs of the native AKHs were tested, whereas 40–70% 
receptor activation was reported with non-amidated analogs of 
the native fly AKH peptides (Caers et al., 2012, 2016). Hence, it 
is most likely that the loss of biological activity in the current 
study is because the deamidated analog and the analog missing 
pGlu were not protected from amino- or carboxypeptidases in 
the hemolymph of C. morosus which may have resulted in the 
peptides being partially digested before reaching the receptor. 
The lack of relevant activity with the N-terminal acetylated-Ala 
analogs in vivo (current study) and in vitro (Caers et al., 2012, 
2016) suggests that the conformation of the resulting peptide 
differs considerably from the native conformation with pGlu, 
preserving proper binding to the AKH receptor. The interaction 
of pGlu with the AKH receptor has been shown to occur in 
the model for AKH ligand-receptor binding in the desert locust, 
Schistocerca gregaria (Jackson et al., 2019). Moreover, the model 
for the RPCH receptor of the water flea, Daphnia pulex, also 
suggests that both termini of this octapeptide are involved in 
binding, i.e., to the extracellular part (Jackson et al., 2018). 

In addition to the termini, the chain length of the ligand 
appears to be critical for the C. morosus AKH receptor: Peram-
CAH-II, one of the octapeptide bioanalogs tested in the present 
study, is identical to the first 8 amino acids of the lead 
peptide, Carmo-HrTH-II, and yet it is not able to achieve a 
potent hypertrehalosemic response; two other octapeptides with 
one amino acid substitution relative to Peram-CAH-II failed 
completely to achieve hypertrehalosemia (see Table 2). The response of C. morosus to Peram-CAH-II is in agreement with a previous study (Gäde and Lohr, 1982) that tested the CC extract of P. americana (thus, containing Peram-CAH-II and also the octapeptide Peram-CAH-I) in the same stick insect bioassay. The poor or no biological activity suggests that the AKH receptor of C. morosus has a weak affinity for octapeptides and that the two amino acids (Thr and Gly) at the C-terminal of the native 
Carmo-HrTH peptides are needed for receptor interaction. This 
is reminiscent of the situation in B. discoidalis where octapeptides 
that differ from the endogenous Bld-HrTH only by one amino 
acid (besides the amino acids Gly-Thr at positions 9 and 
10, of course) are more than 30-fold less active than Bld-
HrTH (Hayes and Keeley, 1990). The relevance of AKH peptide chain length does not seem to be important in an insect like S. gregaria, where two octapeptides and a decapetide (Locmi-AKH-I) are endogenous AKHs. The solution structure of these 
three S. gregaria AKH peptides and models of their binding to the 
endogenous locust receptor were recently reported (Jackson et al., 
2019), wherein it was demonstrated that all three AKHs have the 
same binding site on the S. gregaria AKH receptor, interact with 
similar residues of the receptor and have comparable binding constants. Now that the C. morosus AKH receptor is reportedly 
known from RNA sequencing and a de novo transcriptome 
assembly (Duan Şahbaz and Birgül Iyison, 2019), it opens the 
way for future in vitro receptor assays and molecular modeling of 
ligand-receptor binding to understand why (if, indeed, at all) 
decapetides seem to be favored by the C. morosus AKH receptor. 
As for the decapetide AKH bioanalogs tested in the current 
study, Phyle-CC with Ser10 instead of Thr10 showed the same 
potency as Carmo-HrTH-II (Table 2) suggesting that Thr and 
Ser are interchangeable because both are polar amino acids with 
hydroxylated side chains. The other decapetide bioanalog with 
a single amino acid replacement that was tested in the current study 
is Locmi-AKH-I with Asn3 instead of Thr3; unlike Phyle-CC, 
Locmi-AKH-I could only slightly activate the C. morosus AKH 
receptor. Taking this result together with the fact that Phymo-
AKH, with the double amino acid substitution of Asn3 and Ser10 
instead of the endogenous Thr3 and Thr10 residues, had no 
significant hypertrehalosemic effect (Table 2), it is concluded that 
the Asn residue in position 3 is detrimental for ligand-receptor 
interaction. Even though both amino acids are hydrophilic, the 
difference is the hydroxylated side chain (Thr), whereas the 
second carboxy group of Asn is “neutralized” by an amide 
formation, resulting in a carboxyamide side chain (see Marco 
and Gäde, 2015). The results imply that even the removal of a 
simple side chain, such as a hydroxyl group, can be quite 
crucial for ligand-receptor interaction. However, when Asn3 
appeared in combination with Val2 as in Rommi-CC (instead of 
the endogenous Thr3 and Leu2), hypertrehalosemic action was 
restored to nearly 50% of maximal activity in the current study. 
In Rommi-CC, the hydrophilic-hydrophobic alternating pattern 
is maintained by this double substitution, and having established 
already that the single replacement of Thr3 with Asn3 results in 
little receptor activation (Locmi-AKH-I in the present study), 
these results could mean that the long side chain of Val2 (as 
compared to Leu2) may be the reason for restored activity. Gäde 
(1992) reported that Leu and Val at position 2 of Peram-CAH-II 
can be interchanged without affecting the affinity of the peptide 
for the AKH receptor in P. americana. This seems to be the 
case with the C. morosus AKH receptor too, although we have 
not tested an AKH analog with a single replacement of Val2 for 
Leu2 in the current study. The lack of biological response with 
Locmi-AKH-I in the current study is in agreement with those 
of Gäde and Lohr (1982), and in another case study with the 
stick insect B. extradentatum (that also has Carmo-HrTH-II as an 
endogenous peptide), where Locmi-AKH-I too effected a much 
reduced hypertrehalosemic response in comparison to Carmo-
HrTH-II (Malik et al., 2012). The replacement of Thr3 with Asn3 
in a peptide tested on the moth, H. eson, similarly resulted in no 
biological activity – it should be added that Thr3 is conserved in 
all five endogenous AKH peptides of this moth (Marco and Gäde, 
2015). Thus, clearly the AKH receptors of different species behave 
differently and it appears that co-evolution between endogenous 
peptide(s) and receptor has occurred for the “best fit.” 

It is clear from the bioassay results with various AKH 
bioanalogs that side chains and charge of amino acid residues 
are important for an effective ligand and a biological response. 
Hence, in order to gain greater clarity, we designed an Ala-
replacement series of analogs in the current study starting with 
Ala2 all the way to Ala10 to see how important side chain and 
charge is for the AKH activity of Carmo-HrTH-II in C. morosus. 
Ala was selected as substitution because this is a non-polar 
amino acid and it lacks a hydroxylated side chain. Given that 
Thr has a hydroxylated side chain, it is perhaps not a surprise 
that in this study, the single replacement of Thr at position 3,
5, or 10 of Carmo-HrTH-II, resulted in a complete loss of or a decline in biological activity (Table 3). A contributing factor for the loss of activity is that the substitution of Thr with Ala at position 3 and 5 disrupts the alternating hydrophilic-hydrophobic amino acid pattern of Carmo-HrTH-II (Thr³, Thr⁵, Asn⁷), which in turn might interrupt peptide conformation and thus affect the binding efficacy of the peptide. Previous studies that used in vitro receptor binding assays with expressed AKH receptors of dipteran insects and Ala-replacement analogs of conspecific AKHs that are similar to Carmo-HrTH-II at position 1–4 (Drome-AKH and Glomo-AKH), also showed a lack of receptor activation by analogs where Thr is substituted by an Ala residue (Caers et al., 2012, 2016). Carmo-HrTH-II has two hydrophobic amino acid residues at position 8 and 9 (Trp⁸-Gly⁹) followed by a hydrophilic Thr in position 10; thus, replacing Thr¹⁰ with Ala¹⁰ in the current study increases the C-terminal hydrophobicity of Carmo-HrTH-II, but apparently this is not so crucial as breaking up the alternating hydrophilic-hydrophobic amino acid pattern preceding this hydrophobic tail, for about 50% activity was recorded with the Ala¹⁰ analog of Carmo-HrTH-II (Table 3). This might also mean that the hydroxyl group present on the terminal end (Thr¹⁰) is less important for peptide-receptor interaction in C. morosus compared with that present on non-terminal residues (Thr³, Thr⁵). In a study with modifications of the Thr residues in Loci-AKH-I, Poulos et al. (1994) demonstrated that in L. migratoria, the hydroxyl group of the Thr⁵ of Loci-AK-I is important for biological activity, while that of Thr¹⁰ is not. Similarly, in B. discoidalis a single amino acid change at position 10 was well tolerated without much loss in bioactivity (Ford et al., 1988).

The current study revealed that the substitution of Leu² in Carmo-HrTH-II with Ala has no impact on the AKH receptor on the fat body of C. morosus. Both Leu and Ala residues are non-polar. The only difference between these residues is that Leu has a bulkier alkyl side chain than Ala [-CH₂CH(CH₃)₂ vs. -CH₃]. This might mean that only one methyl group of Leu is involved in the receptor interaction or the whole side chain may, indeed, not be so important. Similarly, in B. discoidalis Ala in position 2, instead of Val in the endogenous Bladi-HrTH, can also be tolerated well (Ford et al., 1988). Finally, two aromatic amino acids are conserved in AKHs and form part of the hallmark features of the AKH peptide family; these are Phe⁴ and Trp⁸. Not surprisingly, therefore, replacement of the aromatic side chains with Ala in the present study resulted in the complete loss of metabolic activity in C. morosus, as has been shown for other insect species too in in vivo and in vitro receptor assays (Ford et al., 1988; Gade and Hayes, 1995; Ziegler et al., 1998; Caers et al., 2012, 2016; Marco and Gade, 2015), thus signifying that these structural features are crucial for receptor-binding in insects. When these aromatic amino acids were swapped to construct a Loci-AK-I with Trp⁴ and Phe⁸, it was not very active in L. migratoria (Velentza et al., 2000). Moreover, single substitutions of Phe⁴ with Trp of Loci-AK-I is tolerated (a 10-fold loss of potency), while the replacement of Trp⁴ with Phe is not (>300 times decrease in potency), leading to the conclusion that position 4 requires a phenyl ring in the side chain, and position 8 an indole ring (Velentza et al., 2000). Ligand interaction diagrams for the two AKH receptor models (S. gregaria; D. pulex) show also clearly that the two aromatic residues in the molecule are essential for binding (Jackson et al., 2018, 2019).

AKHs are predicted to have a β-turn at position 5–8 (Hayes and Keeley, 1990; Zubrycki and Gade, 1994; Cusinato et al., 1998) and, although amino acid sequences and chain length of AKHs can vary, nuclear magnetic resonance experiments assigned turns for each of the examined AKHs (see, for example, Jackson et al., 2019). If this is true for Carmo-HrTH-II as well, then the single replacement of these amino acids with Ala, which results in the removal of the side chain and/or the interruption of alternating hydrophilic pattern, might disrupt the peptide β-conformation. Interrupting the stability of the conformation may hinder the interaction of the peptide with its receptor through the backbone hydrogen bonding (Gade and Hayes, 1995). In the current study, single replacements of amino acids at position 5–9 of Carmo-HrTH-II with Ala resulted in complete loss of potency. This is quite unique. In most studies position 7 could be replaced without major loss of activity (see, for example, Ford et al., 1988; Gade and Hayes, 1995). In B. discoidalis positions 2, 7, and 10 were the ones least affected by single substitution. Unexpectedly the C. morosus AKH receptor did accept the change at position 2 very well (Table 3).

Cardio-Stimulatory Activities in C. morosus

The effect of neuropeptides on the contraction of the dorsal heart was studied before in stick insects, including C. morosus (Ejaz and Lange, 2008; da Silva et al., 2011; Malik et al., 2012; Marco et al., 2018). Although members of the AKH/RPCH family are reported to stimulate the heart beat rate in insects, including stick insects that were either decapitated or ligated behind the head (see Chovański et al., 2016; Marco et al., 2018), it is not known whether these peptides act directly or indirectly on the heart. It is, nevertheless, thought that the stimulation of the heart by the AKH peptides is a mechanism for assisting the faster distribution of fuel metabolites (Gade and Marco, 2013). In the current study we applied the same bioanalog and analogs of Carmo-HrTH-II that were tested in vivo metabolic assays also in a semi-isolated heart assay with C. morosus to see whether the respective biological output could lead to a conclusion about the receptor identity or receptor needs in the two physiological systems.

The application of the N- or C-terminal modified analogs on the C. morosus heart preparations resulted in 58% increase in the frequency of heart contractions compared to that of Carmo-HrTH-II, while the analog that had a pGlu replaced with N-acetyl-Ala did not increase the heart rate. These results indicate that the native peptide may lose some of its binding affinity when the pGlu is removed, or with a free acid at the C-terminus; presumably, the breakdown of the peptides are not as rapid in this assay where the peptides are directly applied to the heart, as opposed to the case of the metabolic assay where the peptides are in the hemolymph and exposed to exopeptidases for 90 min. Further, these results suggest that the peptide analogs with the free termini retain a conformation that can bind to the C. morosus AKH receptor, whereas the peptide conformation brought about...
through the N-acetyl-Ala in position one is not conducive for ligand-receptor binding. The metabolic and the heart assay results in the current study largely indicate the same outcome. In *P. americana*, the affinity is completely lost, with no stimulation of the heart rate when the pGLu or amide is removed in both *in vivo* and semi-isolated heart assays (Baumann et al., 1990).

Single replacements of amino acids with Ala at all positions of Carmo-HrTH-II resulted in the peptide losing its efficacy completely. The data indicate that the side chains of all the amino acids of Carmo-HrTH-II are crucial for eliciting cardio-stimulatory action in *C. morosus*. This is mostly comparable with the data from the metabolic assays *in vivo* where small metabolic changes are measured, while the heart beat is totally unresponsive to the analogs. There is, however, one anomaly that is not easily explained: the Ala2 analog was as active biologically as the lead peptide Carmo-HrTH-II in raising the carbohydrate concentration in the ligated stick insects (Table 3); this same analog did not have a significant effect on the heart contractions in the semi-exposed heart assay (Table 6). We are not able to explain this phenomenon at present.

The myotropic effect measured with the selection of bioanalog yielded largely comparable results to those obtained in the *C. morosus* metabolic assay, with Rommi-CC and Phyle-CC standing out as active peptides, and only a very small effect measured with the other decapetide analogs and the octapeptides.

Since the data trends between the two assays and the peptide analogs tested are so comparable, we conclude that the same receptor is at play in both physiological systems: the rate of heart beat and the mobilization of carbohydrates from the fat body stores in ligated stick insects.

Further studies could be carried out to investigate the mode of action of AKH/RPCH peptides in non-ligated *C. morosus* and other stick insect species since they respond with a biological effect only when the circulation is disrupted between the head and the rest of the body. Wicher et al. (2006) demonstrated that the AKHs of the cockroach *P. americana*, in addition to the metabolic function, act directly on the central nervous system through the release of octopamine from the thoracic dorsal unpaired median (DUM) neurons, and this release of octopamine stimulates locomotor activity. Octopamine is a well-known “fight-or-flight” stress hormone in insects (Verlinden et al., 2010), and its action as heart stimulant has been studied in many insect species (see Chowkiński et al., 2016). Indeed, direct octopaminergic innervation of the insect heart is observed in several insect species, arising from the DUM neurons, and is responsible for cardioacceleratory responses in *D. melanogaster*, *P. americana*, and *M. sexta* (see Johnson et al., 1997; Zornik et al., 1999; Papaethimiou and Theophilidis, 2011). In *C. morosus*, however, octopamine unequivocally inhibits the contraction of the heart of *C. morosus* in a dose-dependent manner, and this is interpreted as an appropriate response in an insect species that relies on cryptic biology to escape predators (Marco et al., 2018). *C. morosus* masquerades as a stick or twig in its habitat, engages in slow movements to keep up the pretense of being part of the food plant, and escapes the interest of predators through thanatosis (i.e., playing dead when detected; Gullan and Cranston, 2014). The Indian stick insect, thus, relies on a strategy of concealment (low energetic costs) in which flight, flight and energy mobilization play no role. By extension, it is expected that the role of stress hormones, such as octopamine and AKHs, would have an opposite physiological effect in such a species.

**Lessons for Green Insecticide Design**

Phasmids are generally not regarded as serious pest insects since they do not pose a direct threat to food security, nor are they known to be vectors of diseases, nevertheless quite damaging outbreaks of stick insect population numbers have been recorded over the years in Australia, North America, China and other geographical areas, where they defoliate economically important timber crops (Baker, 2015). Such defoliated timber trees respond in subsequent years with a smaller stem diameter which is deleterious to the pulp industry. Although many stick insect species are apterous and can therefore not spread as rapidly and widely as winged insects, they can have a serious local impact in the event of an outbreak (Baker, 2015). Pest status notwithstanding, are there any lessons to learn from our work here on the physiological action of AKH ligands in the Indian stick insect *C. morosus* that may be useful for the design of a peptide mimetic that could act specifically to target known pest insects without interfering with other insects?

To date, only a small number of stick insect species have been studied with respect to their AKH neuropeptides. Besides *C. morosus*, primary structures are known from *Sipyloidea sipylos*, *Extatosoma tiaratum*, and *Baculum extradentatus* which all synthesize the decapetide Carmo-HrTH-II (pELTFTPNWGTa) in their CC as the major AKH family neuropeptide, as well as a less abundant neuropeptide that is also biologically active (Gäde, 1989; Gäde and Rinehart, 1990; Malik et al., 2012). In the case of *C. morosus* and *B. extradentatus*, the less abundant peptide was characterized as a post-translational variant of Carmo-HrTH-II, viz. at position 8 the Trp is C-mannosylated (Carmo-HrTH-I, Munte et al., 2008) or modified to kynurenoic acid (Malik et al., 2012). While the structural identity of the additional neuropeptide in *S. sipylos* and *E. tiaratum* was not pursued (due to a lack of sufficient material at the time), we speculate that it may also be Carmo-HrTH-II with a post-translationally modified Trp, and this may be a trait in other stick insects with Carmo-HrTH-II. Recent genomic data sets (Veenstra, 2019) revealed that Carmo-HrTH-II is encoded in the New Zealand stick insect *Citrarchus hookeri* whereas in the evolutionary basal stick insect *Timema christinae* an octapeptide (pEVNFSPSWa) is encoded; this octapeptide is well-known as Anaim-AKH and found in certain dragonflies (Gäde and Marco, 2005) and other basal orders of insects such as Archaeognatha (Marco et al., 2014) and Ephemeroptera (Gäde and Marco, 2012). Carmo-HrTH-II and Anaim-AKH are structurally vastly different peptides – not only in sequence length but also in the actual amino acid sequence. To base a putative lead peptide for AKH insecticide use on the octapeptide would certainly also affect dragonflies which are mostly endangered species, whereas it is envisaged that *C. morosus* will not be targeted by most AKH mimetics as
pesticides because of the very specific needs required for ligand-receptor binding in this species, as deduced here from biological assays: the AKH receptor of *C. morosus* accepts decapeptides and only the amino acids in positions 2 and 10 may deviate from the Carmo-HrTH-II primary sequence.

This seems to be good criteria for specificity but what do we know about the effect of Carmo-HrTH-II on other insects? SAR data only exist from *in vivo* assays and they revealed the following:

1. Carmo-HrTH-II was more than 300-fold less active in the cockroach *B. discoidalis* than the endogenous Bladi-HrTH (Hayes and Keeley, 1990).
2. Carmo-HrTH-II was as, or slightly more active than the endogenous nonapeptide Manse-AKH in the lepidopteran *Manduca sexta* (Fox and Reynolds, 1991; Ziegler et al., 1991).
3. Carmo-HrTH-II was more or less as active in *P. americana* as the endogenous octapeptides Peram-CAH-I and -II (Gäde, 1990).
4. Carmo-HrTH-II was only slightly less active in *L. migratoria* than the endogenous decapeptide Locmi-AKH-I (Gäde, 1990).

Hence, the effect of an insecticidal peptide based on Carmo-HrTH-II would very likely also be effective against some serious pest insects such as a number of lepidopteran larvae, migratory locusts and blattid cockroaches which is not undesirable.

**REFERENCES**

Altstein, M., Ben-Aziz, O., Scheffer, I., Zeltser, I., and Gilon, C. (2000). Advances in the application of neuropeptides in insect control. *Crop. Prot.* 19, 547–555. doi: 10.1016/S0261-2194(00)00071-5

Audsley, N., and Down, R. E. (2015). G protein coupled receptors as targets for next generation pesticides. *Insect. Biochem. Mol. Biol.* 67, 27–37. doi: 10.1016/j.ibmb.2015.07.014

Baker, E. (2015). The worldwide status of phasmids (Insecta: Phasmida) as pests of agriculture and forestry, with a generalised theory of phasmid outbreaks. *Agric. Food Secur.* 4:22. doi: 10.1186/s40066-015-0040-6

Baumann, E., Gäde, G., and Penzlin, H. (1990). Structure-function studies on neurohormone D: activity of naturally-occurring hormone analogues. *J. Comp. Physiol. B* 160, 423–429. doi: 10.1007/BF01075674

Bideya, S. S., Bockemühl, T., and Büschges, A. (2018). Six-legged walking in insects: how CPGs, peripheral feedback, and descending signals generate coordinated and adaptive motor rhythms. *J. Neurophysiol.* 119, 459–475. doi: 10.1152/jn.00658.2017

Burn, A. J., Coaker, T. H., and.Jepson, P. C. (1987). *Integrated Pest Management*. London: Academic Press.

Caers, J., Janssen, T., Van Rompay, L., Broeckx, V., Van Den Abbeele, J., Gäde, G., et al. (2016). Characterization and pharmacological analysis of two adipokinetic hormone receptor variants of the tsetse fly, *Glossina morsitans morsitans*. *Insect Biochem. Mol. Biol.* 70, 73–84. doi: 10.1016/j.ibmb.2015.11.010

Caers, J., Peeters, L., Janssen, T., De Haes, W., Gäde, G., and Schoofs, L. (2012). Structure-activity studies of Drosophila adipokinetic hormone (AKH) by a cellular expression system of dipteran AKH receptors. *Gen. Comp. Endocrinol.* 177, 332–337. doi: 10.1016/j.ygecen.2012.04.025

Capinera, J. L. (2010). *Insects and Wildlife: Arthropods and their Relationships with Wild Vertebrate Animals*. Chichester: Wiley-Blackwell.

**DATA AVAILABILITY STATEMENT**

All datasets generated for this study are included in the article/supplementary material.

**AUTHOR CONTRIBUTIONS**

HM and GG conceptualized the research project, financed the work, and supervised OK. OK and HM performed experiments, reared the animal cultures, and analyzed the data. GG contributed chemicals and peptides, helped with interpretation of the data, and writing the draft manuscript. OK performed most biological assays, was involved in data interpretation, and drafting of the manuscript. HM helped with interpretation and analyses of data, writing, and refining the draft manuscript. All the authors agreed to be accountable for the content of the work presented here.

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Chowaniak, S., Lubawy, J., Urbanski, A., and Rosinski, G. (2016). Cardioregulatory functions of neuropeptides and peptide hormones in insects. *Protein Peptide Lett.* 23, 913–931.

Cusinato, O., Drake, A. F., Gäde, G., and Goldsworthy, G. J. (1998). The molecular conformations of representative arthropod adipokinetic peptides determined by circular dichroism spectroscopy. *Insect Biochem. Mol. Biol.* 28, 43–50. doi: 10.1016/S0261-2194(97)00094-5

da Silva, S. R., da Silva, R., and Lange, A. B. (2011). Effects of crustacean cardioactive peptide on the hearts of two Orthopteran insects, and the demonstration of a Frank-Starling-like effect. *Gen. Comp. Endocrinol.* 171, 218–224. doi: 10.1016/j.ygcen.2011.01.015

Duan Şahbaz, B., and Birgil Ilyison, N. (2019). Prediction and expression analysis of G protein-coupled receptors in the laboratory stick insect, *Carausius morosus*. *Turk. J. Biol.* 43, 77–88. doi: 10.3906/biy-1809-27

Ejaz, A., and Lange, A. B. (2008). Peptidergic control of the heart of the stick insect, *Bucaclum extradentatum*. *Peptides* 29, 214–225.

Ford, M. M., Hayes, T. K., and Keeley, L. L. (1988). “Structure-activity relationships for insect hypertrehalosaeic hormone: the importance of side chains and termini,” in Peptides. *Chemistry and Biology*, ed. G. M. Marshall (Leiden: Escom Press), 653–655.

Fox, A. M., and Reynolds, S. E. (1991). The pharmacology of the lipid-mobilizing response to adipokinetic hormone family peptides in the moth, *Manduca sexta*. *J. Insect Physiol.* 37, 373–381.

Gäde, G. (1979). Adipokinetic and hyperglycaemic factor(s) in the corpora cardiaca/corpora allata complex of the stick insect, *Carausius morosus*. I. Initial characteristics. *Physiol. Entomol.* 4, 131–134.

Gäde, G. (1985). Isolation of the hypertrehalosaeic factors I and II from the corpus carduacum of the Indian stick insect, *Carausius morosus*, by reversed-phase high-performance liquid chromatography, and amino-acid composition of factor II. *Biol. Chem.* 366, 195–200. doi: 10.1515/bchm3.1985.366.1.195
Gäde, G. (1986). Relative hypertrehalosaemic activities of naturally occurring neuropeptides from the AKH/RPCH family. Z. Naturforsch. 41c, 315–320. doi: 10.1515/znc-1986-0312

Gäde, G. (1989). Isolation, physiological characterization, release and sequence elucidation of a hypertrehalosaemic neuropeptide from the corpus cardiacum of the stick insect, Stylodidea sylphus. Physiol. Entomol. 14, 405–418.

Gäde, G. (1990). Structure-function studies on hypertrehalosaemic and adipokinetic hormones: activity of naturally occurring analogues and some N- and C-terminal modified analogues. Physiol. Entomol. 15, 299–316. doi: 10.1111/j.1365-3032.1990.tb00518.x

Gäde, G. (1992). Structure-activity relationships for the carbohydrate-mobilizing action of further bioanalogues of the adipokinetic hormone/red pigment-concentrating hormone family of peptides. J. Insect Physiol. 38, 259–266. doi: 10.1016/0022-1910(92)90025-M

Gäde, G. (1993). Structure-activity relationships for the lipid-mobilizing action of further bioanalogues of the adipokinetic hormone/red pigment-concentrating hormone family of peptides. J. Insect Physiol. 39, 375–383. doi: 10.1016/0022-1910(93)90025-M

Gäde, G. (1997). “The explosion of structural information on insect neuropeptides,” in Fortschritte der Chemie organischer Naturstoffe/Progress in the Chemistry of Organic Natural Products, eds W. Hertz, G. W. Kirby, R. E. Moore, W. Steglich, and C. Tamm (New York, NY: Springer), 1–128.

Gäde, G. (2005). “Adipokinetic hormones: a new take on biodiversity,” in Biologically Active Peptides, ed. A. Kastin (San Diego: Elsevier Inc), 185–190.

Gäde, G. (2015). Structure-activity relationships of adipokinetic hormone analogs in the striped hawk moth, Hipposphora eson. Peptides 68, 205–210.

Gäde, G., and Marco, H. G. (2020). “Adipokinetic Hormone: A Hormone for All Seasons?,” in Advances in Invertebrate (Neuro)Endocrinology, Vol. 2. Arthropoda, eds S. Saleuddin, A. B. Lange, and I. Orchard (Palm Bay, FL: Apple Academic Press).

Gäde, G., Kellner, R., and Gäde, G. (1990). Structure–function studies on hypertrehalosaemic and cardiacum of the stick insect, Carausius morosus. Z. Physiol. Entomol. 17, 2192–2204. doi: 10.1016/0212-5062(91)90135-4

Jackson, G. E., Pavadai, E., Gäde, G., and Andersen, N. H. (2019). The adipokinetic hormones and their cognate receptor from the desert locust, Schistocerca gregaria: solution structure of endogenous peptides and models of their binding to the receptor. PeerJ 7:e7514. doi: 10.7717/peerj.7514
Stone, J. V., Mordue, W., Broomfield, C. E., and Hardy, P. M. (1978). Structure-activity relationships for the lipid-mobilising action of locust adipokinetic hormone. Eur. J. Biochem. 89, 195–202.

Veenstra, J. A. (2019). Two Lys-vasopressin-like peptides, EF-Lamide, and other phasmid neuropeptides. Gen. Comp. Endocrinol. 278, 3–11.

Velentza, A., Spiliou, S., Poulos, C. P., and Goldsworthy, G. J. (2000). Synthesis and biological activity of adipokinetic hormone analogues with modifications in the 4–8 region. Peptides 21, 631–637.

Verlinden, H., Vleugels, R., Marchal, E., Badisco, L., Pflüger, H. J., Blenau, W., et al. (2010). The role of octopamine in locusts and other arthropods. J. Insect Physiol. 56, 854–867.

Verlinden, H., Vleugels, R., Zels, S., Dillen, S., Lenaerts, C., Crabbe, K., et al. (2015). Receptors for neuronal or endocrine signalling molecules as potential targets for the control of insect pests. Adv. Insect Physiol. 46:167.

Wahedi, A., Gäde, G., and Paluzzi, J.-P. (2019). Insight into mosquito GnRH-related neuropeptide receptor specificity revealed through analysis of naturally occurring and synthetic analogs of this neuropeptide family. Front. Endocrinol. 10:742. doi: 10.3389/fendo.2019.00742

Wicher, D., Agricola, H. J., Sohler, S., Gundel, M., Heinemann, S. H., Wollweber, L., et al. (2006). Differential receptor activation by cockroach adipokinetic hormones produces differential effects on ion currents, neuronal activity, and locomotion. J. Neurophysiol. 95, 2314–2325.

Ziegler, R., Cushing, A. S., Walpole, P., Miromoto, H., and Jasensky, R. D. (1998). Analogs of Manduca adipokinetic hormone tested in a bioassay and in a receptor-binding assay. Peptides 19, 481–486.

Ziegler, R., Eckart, K., Jasensky, R. D., and Law, J. H. (1991). Structure-activity studies on adipokinetic hormones in Manduca sexta. Arch. Insect Biochem. Physiol. 18, 229–237.

Zornik, E., Paisley, K., and Nichols, R. (1999). Neural transmitters and a peptide modulate Drosophila heart rate. Peptides 20, 45–51.

Zubrzycki, I. Z., and Gäde, G. (1994). Conformational study on an insect neuropeptide of the AKH/RPCH-family by combined 1H-NMR spectroscopy and molecular mechanics. Biochem. Biophys. Res. Commun. 198, 228–235.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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