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RESEARCH ARTICLE

CCAP and FMRFamide-like peptides accelerate the contraction rate of the antennal accessory pulsatile organs (auxiliary hearts) of mosquitoes

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ABSTRACT

Insects rely on specialized accessory pulsatile organs (APOs), also known as auxiliary hearts, to propel hemolymph into their antennae. In most insects, this is accomplished via the pulsations of a pair of ampulla located in the head, each of which propels hemolymph across an antenna via an antennal vessel. Once at the distal end of the appendage, hemolymph returns to the head via the antennal hemocoel. Although the structure of the antennal hearts has been elucidated in various insect orders, their hormonal modulation has only been studied in cockroaches and other hemimetabolous insects within the superorder Polyneoptera, where proctolin and FMRFamide-like peptides accelerate the contraction rate of these auxiliary hearts. Here, we assessed the hormonal modulation of the antennal APOs of mosquitoes, a group of holometabolous (Endopterygota) insects within the order Diptera. We show that crustacean cardioactive peptide (CCAP), FMRFamide and SALDKNFMRFamide increase the contraction rate of the antennal APOs and the heart of *Anopheles gambiae*. Both antennal hearts are synchronously responsive to these neuropeptides, but their contractions are asynchronous with the contraction of the heart. Furthermore, we show that these neuropeptides increase the velocity and maximum acceleration of hemolymph within the antennal space, suggesting that each contraction is also more forceful. To our knowledge, this is the first report demonstrating that hormones of a holometabolous insect modulate the contraction dynamics of an auxiliary heart, and the first report that shows that the hormones of any insect accelerate the velocity of hemolymph in the antennal space.

KEY WORDS: Dorsal vessel, Hemolymph, Hemocoel, Neuropeptide, Diptera, *Anopheles gambiae*

INTRODUCTION

Insects have an open circulatory system that transports nutrients, waste, hormones, immune factors and other molecules to all regions of the body (Chapman et al., 2013; Klowden, 2013; Wirkner et al., 2013; Hillyer, 2015). This circulatory system is composed of hemolymph (insect blood), the hemocoel (open body cavity) and several contractile pumps. The primary pump is a muscular tube called the dorsal vessel. This vessel extends the length of the insect along the dorsal midline, and is divided into a contractile heart in the abdomen and the aorta in the thorax. In addition to the dorsal vessel, insects employ accessory pulsatile organs (APOs), also called auxiliary hearts, to propel hemolymph into narrow areas of the body, or areas that are distant from the dorsal vessel (Pass, 2000; Pass et al., 2006). Depending on the species, these APOs are located at the base of the antennae, wings and ovipositor, and in the ventral abdomen (Pass, 1991; Richter and Hertel, 1997; Matus and Pass, 1999; Andereck et al., 2010; Boppana and Hillyer, 2014; Hustert et al., 2014; Wipfler and Pass, 2014; Pass et al., 2015).

Antennal accessory pulsatile organs are present in nearly all insects (Pass, 2000; Pass et al., 2006). They display significant structural variability across taxa, but the general morphology of an antennal APO includes an ampulla in the head, an antennal vessel that originates in the ampulla and extends into the antenna, and an antennal hemocoel. Some antennal APOs propel hemolymph via the relaxation of the ampulla, while others do so via the contraction of the ampulla or do not contract at all (Pass, 1991, 2000; Matus and Pass, 1999; Pass et al., 2006). Although the structure of the antennal APOs has been elucidated in multiple insect species, less is known about endogenous factors that control their contraction. In cockroaches and some polyneopterans (lower Neoptera), the neuropeptide proctolin and three FMRFamide-like peptides (FLPs) accelerate the contraction rate of the antennal APOs, whereas the neuropeptide allatostatin and another FLP do not (Hertel et al., 1985, 1997, 2012; Hertel and Penzlin, 1992; Lange et al., 1993; Predel et al., 2004). Whether these or other factors accelerate the contraction rate of the antennal APOs of non-polyneopteran insects remains unknown; however, proctolin and certain FLPs are cardioacceleratory in diverse insect species (Cuthbert and Evans, 1989; Robb and Evans, 1990; Zornik et al., 1999; Sliwowska et al., 2001; Nichols, 2006; Ejaz and Lange, 2008; Hillyer et al., 2014). Thus, it is likely that other cardiomodulatory factors affect the contraction rate of the antennal APOs of insects.

In mosquitoes, the relaxation of the ampulla of each APO propels hemolymph into the antenna via the antennal vessel. After hemolymph reaches the distal end of this appendage, it flows back to the head via the antennal hemocoel (Clements, 1956; Sun and Schmidt, 1997; Boppana and Hillyer, 2014). Intravital imaging experiments that visualized the flow of hemolymph confirmed these findings, and demonstrated that hemolymph is propelled to the distal end of the antennae at a velocity that is four times faster than the velocity in which it flows back toward the head (Boppana and Hillyer, 2014). Although no investigations have tested the effect of endogenous factors on the contraction rate of the antennal APOs of mosquitoes, the neuropeptides CCAP, FMRFamide and SALDKNFMRFamide, and the neurotransmitters serotonin and glutamate, have been shown to accelerate the heart rate of these insects (Estevez-Lao et al., 2013; Hillyer et al., 2014, 2015). In the present study, we tested the effect of CCAP, FMRFamide and

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SALDKNFMRFamide on the antennal APOs of the mosquito *Anopheles gambiae*, and show that these factors increase both the contraction rate of auxiliary hearts and the velocity of hemolymph in the antennal space.

MATERIALS AND METHODS

Mosquito rearing

Anopheles gambiae Giles *sensu stricto* (G3 strain; Diptera: Culicidae) were reared as described elsewhere (Estevez-Lao et al., 2013). Briefly, eggs were hatched in water, larvae were fed a mix of koi food and baker's yeast, and adults were fed a 10% sucrose solution. Mosquito rearing and maintenance were performed in an environmental chamber held at 27°C and 75% relative humidity, under a 12 h:12 h light:dark photoperiod. All experiments were performed on female adult mosquitoes at 5 days post-eclosion.

Visualization and measurement of mosquito antennal APO and heart contractions

Mosquitoes were anesthetized by placing them in a −20°C freezer for 60 s, and then kept on a Petri dish on ice. The general procedure used to inject and visualize the contractions of the antennal APOs and the heart was essentially as we have described previously (Boppana and Hillyer, 2014). Briefly, under a Nikon SMZ645 stereomicroscope (Nikon, Tokyo, Japan), a mosquito was injected with ∼0.2 µl of 0.1% solids 1 µm diameter neutral density yellow-green (505/515) carboxylate-modified fluorescent microspheres (Molecular Probes, Eugene, OR, USA) in either phosphate buffered saline (PBS; pH 7.0; used as the vehicle solution) or in PBS containing CCAP (H-PFCNAFTGC-NH_2, including a disulfide bond between the cysteines), FMRFamide (H-FMRF-NH_2) or SALDKNFMRFamide (H-SALDKNFMRF-NH_2) at a concentration of 1×10^{-6} mol l^{-1} (all peptides were purchased from Bachem Americas, Torrance, CA, USA). After injection, the legs and wings were removed using a razor blade, and the mosquito was placed dorsal side up on a glass slide between two rolled pieces of Parafilm (Pechiney Plastic Packaging Company, Chicago, IL, USA), and with the head on top of a flat piece of Parafilm. The slide was then transferred to a Nikon 90i compound microscope equipped with a Nikon Intensilight C-HGFI fluorescence illumination unit, a Photometrics CoolSNAP HQ2 camera (Roper Scientific, Ottobrunn, Germany) and Nikon Advanced Research NIS-Elements software. At 10 min post-injection, a 60 s intravital video of both antennal APOs was taken using low level fluorescence illumination (achieved by placing an ND 8 filter in the light path), a 40× objective, and camera settings of 3×3 binning, 4× gain and the 1-frame exposure setting. Immediately after, a second 60 s video of the heart was taken through the dorsal abdomen using identical parameters, except that a 10× objective was used.

Contractions of each antennal APO were counted manually by visualizing the contraction-driven movement of the fluorescent microspheres that had aggregated at each APO (Boppana and Hillyer, 2014). Contractions of the heart were counted manually by annotating each wave-like contraction of cardiac muscle that shifted the fluorescent microspheres that had aggregated at the periostial regions of the heart (King and Hillyer, 2012; Boppana and Hillyer, 2014). For CCAP, six independent trials were conducted, each containing between three and five mosquitoes per treatment (n=27 for both CCAP and PBS). For FMRFamide, eight independent trials were conducted, each containing between two and five mosquitoes per treatment (n=27 for FMRFamide; n=30 for PBS). For SALDKNFMRFamide, 11 independent trials were conducted, each containing between two and five mosquitoes per treatment (n=29 for SALDKNFMRFamide; n=26 for PBS). For descriptions on the structural mechanics of the antennal APOs and the heart of mosquitoes, including videos of their contraction and hemolymph flow, see our previously published work (Andereck et al., 2010; Glenn et al., 2010; Boppana and Hillyer, 2014; League et al., 2015).

Measurement of hemolymph velocity in the antennal space

The general procedure used to measure hemolymph velocity in the antennae was essentially as we have described previously (Boppana and Hillyer, 2014). Briefly, under a Nikon SMZ645 stereomicroscope, an anesthetized mosquito was injected with PBS, or with PBS containing CCAP, FMRFamide or SALDKNFMRFamide at 1×10^{-6} mol l^{-1}. Ten minutes later, the mosquito was injected with ∼0.2 µl 0.02% solids, 0.5 µm diameter, neutral density, yellow-green (505/515) carboxylate-modified fluorescent microspheres (Molecular Probes) in PBS. The legs were then removed, the mosquito was placed ventral side up on a glass slide, a rolled piece of Parafilm was placed on top of each wing, and the head was placed on a flat piece of Parafilm. The slide was then transferred to the Nikon 90i microscope ensemble described above, and a 60 s intravital video of the antennae was taken using low level fluorescence illumination (achieved by placing an ND 4 filter in the light path), a 10× objective, and camera settings of 2×2 binning, 4× gain and the 1-frame exposure setting.

Hemolymph velocity was determined by measuring the rate at which the neutral density microspheres travelled across the antennal space. For this, the manual feature of the Object Tracker module of NIS-Elements was used to quantitatively track the trajectory of individual microspheres as they flowed up or down the antennae, and the distance traveled, the velocity and the maximum acceleration of each microsphere were calculated. Six independent trials were conducted, each containing four or five mosquitoes per treatment. A total of 15, 17, 16 and 15 mosquitoes were analyzed for CCAP, FMRFamide, SALDKNFMRFamide and PBS (vehicle control), respectively. For each mosquito, three microspheres were tracked as they moved up an antenna and three microspheres were tracked as they moved down an antenna. Microspheres were tracked for an average distance of 359 µm (57 µm s.d.) and 340 µm (59 µm s.d.) in the upward and downward directions, respectively.

Statistical analysis

Data on basal contraction rates were analyzed using the Friedman test, followed by Dunn's multiple comparisons *post hoc* test. Data on the effect of neuropeptides on the contraction rate of each antennal APO or the heart were analyzed using the Mann–Whitney test. Correlation analyses were performed by plotting the individual contraction rate of two pulsatile organs from the same mosquito, and then calculating the correlation coefficient (R) as well as the Pearson correlation P-value of the entire dataset. When the antennal APO contraction data were correlated to the heart contraction data, the contraction rates from the two antennal APOs (which are nearly the same) were averaged and plotted against the heart value of that individual. Data on hemolymph velocity and maximum acceleration were transformed using the equation $Y=\ln(Y)$, and the transformed data were analyzed by ANOVA, followed by Tukey's multiple comparisons *post hoc* test.

RESULTS

Antennal APO and heart contraction rates

Analysis of mosquitoes that were injected with the vehicle control (a neuropeptide was not injected) revealed that both the left and the right antennal APOs contract at an average rate of 1.09 Hz, and that the heart contracts at an average rate of 1.94 Hz (Fig. 1A).

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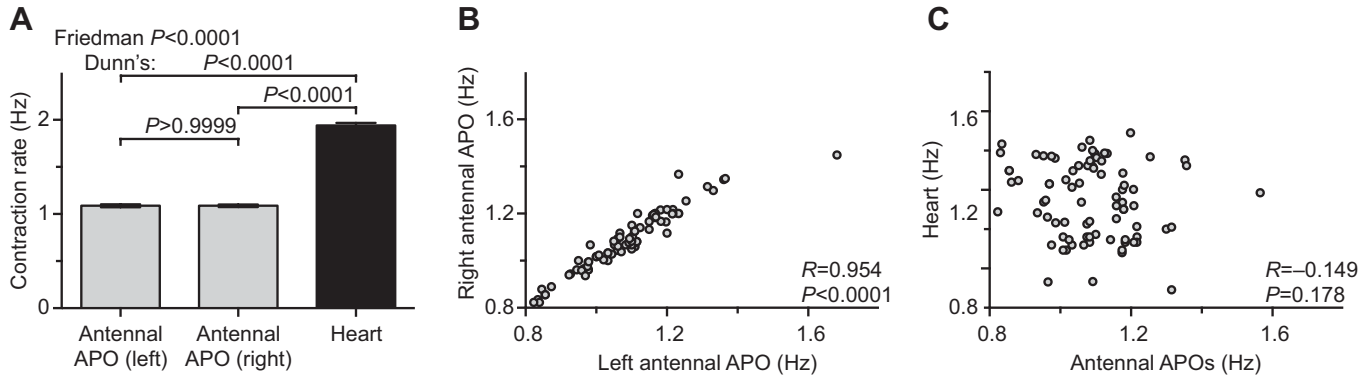

Fig. 1. Resting state contraction rates of the antennal accessory pulsatile organs (APOs) and the heart of mosquitoes. (A) Average contraction rates of the antennal APOs and the heart of mosquitoes treated with the vehicle control. Error bars represent ±s.e.m. *P*-values result from a Friedman test and Dunn's *post hoc* tests. (B,C) Correlation graphs comparing the contraction rate of the two antennal APOs of individual mosquitoes (B) and comparing the contraction rate of the antennal APOs with the contraction rate of the heart (C). For B and C, *R*-values are the correlation coefficients, *P*-values result from Pearson correlation tests, and each data point represents an individual mosquito (*n*=83).

Statistical analyses of the data using the Friedman test detected a difference between the contraction rates of the pulsatile organs ($P<0.0001$), which is because the heart contracts at a higher rate than the antennal APOs (Dunn's $P<0.0001$ for both comparisons). Correlation analyses confirmed that both antennal APOs contract in synchrony ($R=0.954$, Pearson $P<0.0001$, slope=0.89; Fig. 1B), but there is no synchrony between the contraction rates of the antennal APOs and the heart ($R=-0.149$, Pearson $P=0.178$; Fig. 1C). These resting state (basal) data are in agreement with our previously published work (Boppana and Hillyer, 2014).

CCAP, FMRFamide and SALDKNFMRFamide increase the antennal APO and heart contraction rates

Previous studies have shown that the neuropeptides CCAP, FMRFamide and SALDKNFMRFamide accelerate the heart contraction rate of mosquitoes (Estevez-Lao et al., 2013; Hillyer et al., 2014). This occurs in a dose-dependent manner, and for all three peptides, the maximum effect was seen when the peptide was injected at approximately 1×10^{-6} mol l^{-1}. Thus, to determine whether these cardioacceleratory peptides also accelerate the antennal APOs, mosquitoes were injected with a vehicle solution or with a vehicle solution containing one of these neuropeptides at a

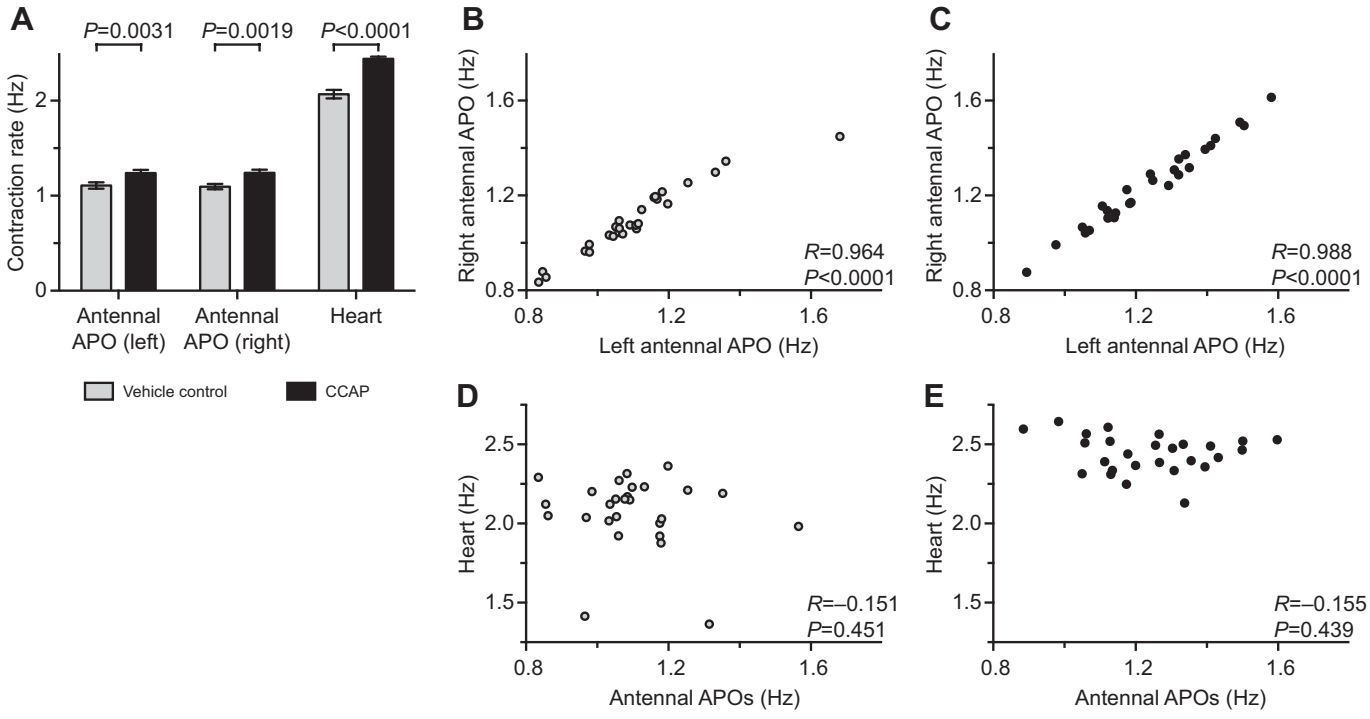

Fig. 2. Effect of CCAP on the contraction rates of the antennal APOs and the heart of mosquitoes. (A) Average contraction rates of the antennal APOs and the heart of mosquitoes treated with a vehicle control or with CCAP. Error bars represent ±s.e.m. *P*-values result from Mann–Whitney tests. (B,C) Correlation graphs comparing the contraction rate of the two antennal APOs of mosquitoes treated with the vehicle control (B) or with CCAP (C). (D,E) Correlation graphs comparing the contraction rate of the antennal APOs with the contraction rate of the heart of mosquitoes treated with the vehicle control (D) or with CCAP (E). For B–E, *R*-values are the correlation coefficients, *P*-values result from Pearson correlation tests, and each data point represents an individual mosquito (*n*=27 for vehicle control and CCAP).

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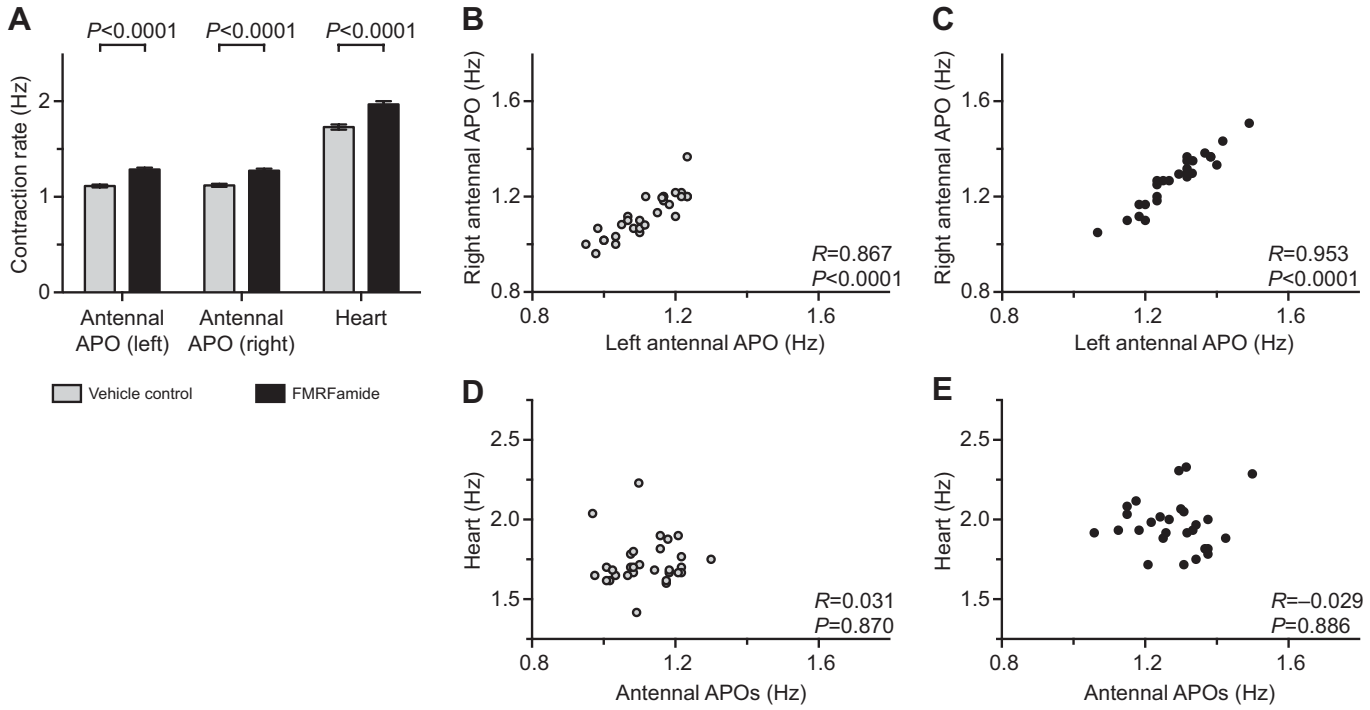

Fig. 3. Effect of FMRFamide on the contraction rates of the antennal APOs and the heart of mosquitoes. (A) Average contraction rates of the antennal APOs and the heart of mosquitoes treated with a vehicle control or with FMRFamide. Error bars represent ±s.e.m. *P*-values result from Mann–Whitney tests. (B,C) Correlation graphs comparing the contraction rate of the two antennal APOs of mosquitoes treated with the vehicle control (B) or with FMRFamide (C). (D,E) Correlation graphs comparing the contraction rate of the antennal APOs with the contraction rate of the heart of mosquitoes treated with the vehicle control (D) or with FMRFamide (E). For B–E, *R*-values are the correlation coefficients, *P*-values result from Pearson correlation tests, and each data point represents an individual mosquito (n=30 for vehicle control and 27 for FMRFamide).

concentration of 1×10^{-6} mol l^{-1}. The contraction rates of both the antennal APOs and the heart were then measured.

Analysis of the heart contraction data showed that, relative to the vehicle controls, treatment of mosquitoes with CCAP, FMRFamide and SALDKNFMRFamide accelerated the heart contraction rate by 18, 14 and 15%, respectively (Mann–Whitney $P<0.0001$ for all; Figs 2A, 3A and 4A). Similarly, treatment of mosquitoes with these neuropeptides significantly increased the contraction rate of the left antennal APO by 12, 16 and 21%, respectively, and the right antennal APO by 13, 14 and 21%, respectively ($P\leq0.0031$ for all; Figs 2A, 3A and 4A).

Analysis of all control mosquitoes revealed a strong correlation between the contraction rate of the left antennal APO and the contraction rate of the right antennal APO (Fig. 1B), and this correlation was similar when the three sets of control mosquitoes were analyzed independently ($R\geq0.867$ for all, Pearson $P<0.0001$ for all; Figs 2B, 3B and 4B). Following treatment with CCAP, FMRFamide or SALDKNFMRFamide, a strong correlation was also observed between the contraction rates of the left and right antennal APOs ($R\geq0.953$ for all, Pearson $P<0.0001$ for all; Figs 2C, 3C and 4C), and together with slope values that approach 1, this indicates that regardless of the treatment, the two antennal APOs contract in synchrony.

When the correlation analysis compared the heart contraction rate with the antennal APO contraction rate, no correlation was observed between the datasets of control mosquitoes, regardless of whether they were analyzed together (Fig. 1C), or whether the three sets of control mosquitoes were analyzed independently ($-0.151\leq R\leq0.45$, Pearson $P\geq0.451$; Figs 2D, 3D and 4D). Similarly, no correlation was observed between the heart contraction rate and the antennal APO contraction rate of mosquitoes that had been treated with any of the neuropeptides ($-0.155\leq R\leq-0.029$, Pearson $P\geq0.439$; Figs 2E, 3E and 4E). Together, these data indicate that, regardless of the treatment, the antennal APOs and the heart do not contract in synchrony.

Visual comparison of the correlation graphs further illustrates the acceleratory nature of CCAP, FMRFamide or SALDKNFMRFamide on both the antennal APOs and the heart (Figs 2B–E, 3B–E and 4B–E). That is, comparison of the vehicle control graphs with the neuropeptide graphs shows a marked upward and rightward shift in the data, graphically illustrating that treatment induces an increase in the contraction rate of these pulsatile organs.

CCAP, FMRFamide and SALDKNFMRFamide increase hemolymph flow velocity in the antennal space

In mosquitoes treated with the vehicle solution, hemolymph flowed from the head to the distal end of the antenna (upward direction) via the antennal vessel at an average velocity of 167 μm s^{-1}, and then flowed back toward the head (downward direction) via the antennal hemocoel at an average velocity of 48 μm s^{-1} (Fig. 5A–C). Following the same trend, the maximum acceleration of hemolymph was 7132 μm s^{-2} when flowing in the upward direction and 3164 μm s^{-2} when flowing in the downward direction (Fig. 5D,E). These values are in agreement with our previous work, and the differences in upward versus downward values are due to the structure of the antennal space: hemolymph is pumped upward via a narrow antennal vessel and downward via a wider antennal hemocoel (Fig. 5A) (Boppana and Hillyer, 2014).

Treatment of mosquitoes with CCAP, FMRFamide and SALDKNFMRFamide increased the velocity of hemolymph by 32,

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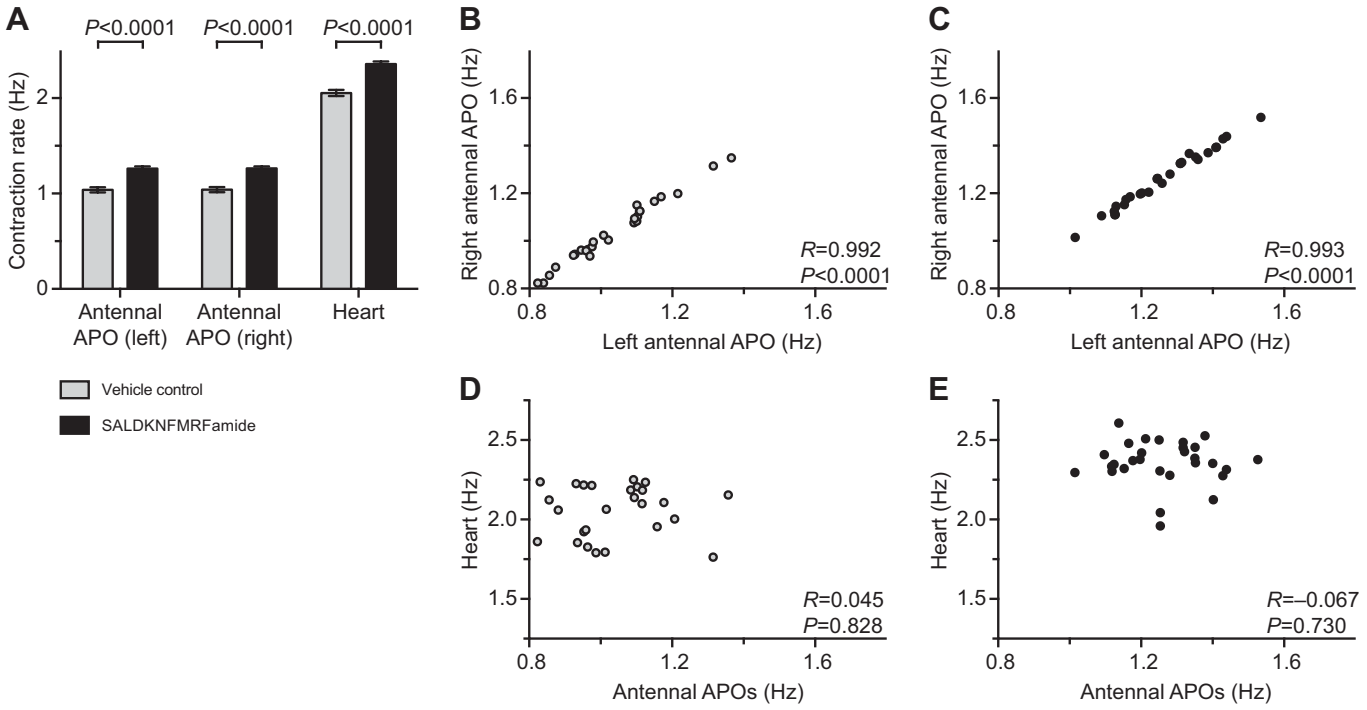

Fig. 4. Effect of SALDKNFMRFamide on the contraction rates of the antennal APOs and the heart of mosquitoes. (A) Average contraction rates of the antennal APOs and the heart of mosquitoes treated with a vehicle control or with SALDKNFMRFamide. Error bars represent ±s.e.m. *P*-values result from Mann–Whitney tests. (B,C) Correlation graphs comparing the contraction rate of the two antennal APOs of mosquitoes treated with the vehicle control (B) or with SALDKNFMRFamide (C). (D,E) Correlation graphs comparing the contraction rate of the antennal APOs with the contraction rate of the heart of mosquitoes treated with the vehicle control (D) or with SALDKNFMRFamide (E). For B–E, *R*-values are the correlation coefficients, *P*-values result from Pearson correlation tests, and each data point represents an individual mosquito (*n*=26 for vehicle control and 29 for SALDKNFMRFamide).

33 and 21% when traveling in the upward direction, respectively, and by 32, 23 and 7% when traveling in the downward direction, respectively (Fig. 5B,C). All three neuropeptides significantly increased the velocity of hemolymph in the upward direction, but only CCAP significantly increased hemolymph velocity in the downward direction. When the maximum acceleration of hemolymph was considered, CCAP, FMRFamide and SALDKNFMRFamide increased this value by 31, 31 and 33% when traveling in the upward direction, respectively, and by 22, 25 and 7% when traveling in the downward direction, respectively (Fig. 5D,E). Neuropeptide-induced increases in the maximum acceleration were statistically significant when hemolymph traveled in the upward direction, but were not significant in the downward direction. Together, these data suggest that these myotropic neuropeptides induce contractions that are both more frequent and more forceful.

DISCUSSION

The antennae of insects are narrow appendages that detect chemical, tactile, thermal and auditory stimuli (Chapman and Simpson, 2013). They also function during the mating of fleas, and as part of the sun compass that drives the autumnal migration of North American monarch butterflies (Hsu and Wu, 2001; Merlin et al., 2009). The antennae of insects are most often long and narrow appendages, and as such, simple diffusion is insufficient to deliver hemolymph to all of their regions. For that reason, the vast majority of insects employ APOs located near the base of the antennae to supplement the circulatory currents created by the contraction of the dorsal vessel. The structure of the antennal APOs has been elucidated in multiple insect orders, but the most comprehensive studies have focused on cockroaches (order Blattodea). Furthermore, until the present study, the hormonal modulation of antennal APO contraction had only been studied in cockroaches and other polyneopterans (Hertel et al., 2012), with no studies focusing on the largest group of insects: the Endopterygota (Holometabola). Thus, we assessed whether the neurohormones CCAP, FMRFamide and SALDKNFMRFamide affect the physiology of the antennal APOs of mosquitoes (holometabolous insects in the order Diptera), and demonstrate that these three neuropeptides increase the contraction rate of these pulsatile organs in a manner that is similar to how they accelerate the heart. Furthermore, we show that these neuropeptides also increase the velocity and maximum acceleration of hemolymph in the antennal space.

In cockroaches, two types of neuropeptides are known to accelerate the antennal APO contraction rate: proctolin and three FLPs (Hertel et al., 1985, 1997, 2012; Hertel and Penzlin, 1992; Lange et al., 1993; Predel et al., 2004). Proctolin stimulates the contraction of visceral, skeletal and cardiac muscle, and modulates digestion, egg laying, and both sexual and feeding behaviors (Konopinska and Rosinski, 1999; Isaac et al., 2004; Ejaz and Lange, 2008). However, a search of the genomes of *A. gambiae*, *Aedes aegypti* and *Culex quinquefasciatus* (via www.vectorbase.org) reveals that neither proctolin nor the proctolin receptor is encoded in the mosquito lineage. FMRFamide-like peptides, however, are produced in mosquitoes (Predel et al., 2010; Siju et al., 2014). In *A. gambiae*, the FMRFamide gene is alternatively spliced and encodes eight FLPs (Hillyer et al., 2014). One of these peptides, SALDKNFMRFamide, contains an FMRFamide sequence at its C terminus, and both SALDKNFMRFamide and FMRFamide accelerate the heart rate of *A. gambiae* (Hillyer et al., 2014). However, the cardioacceleratory properties of FLPs are not

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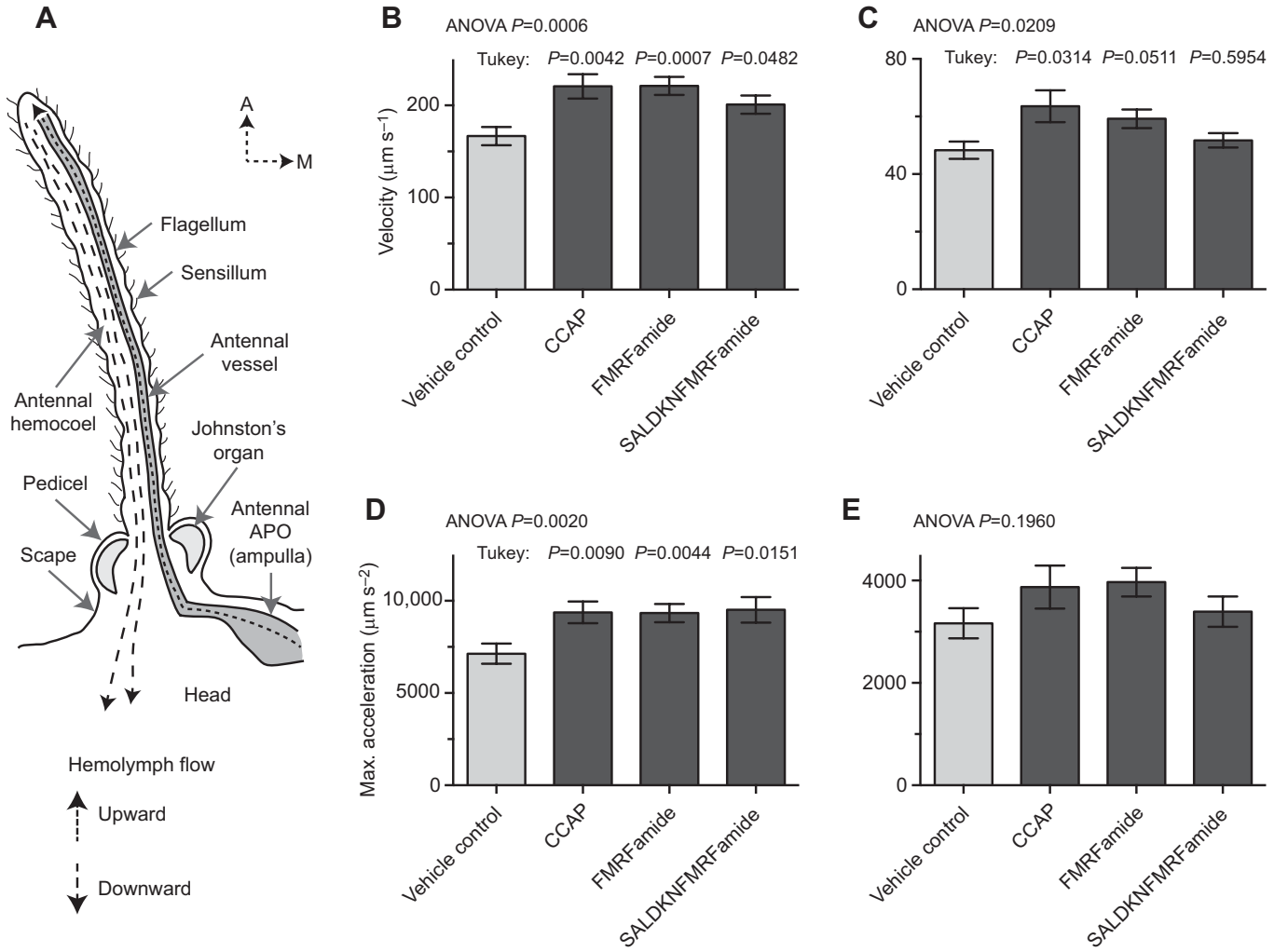

Fig. 5. Effect of CCAP, FMRFamide and SALDKNFMRFamide on hemolymph flow velocity and maximum acceleration in the antennal space. (A) Diagrammatic representation of hemolymph flow in the antennal space (left antenna, dorsal view). The ampulla of an antennal APO propels hemolymph in the upward direction via an antennal vessel. When hemolymph reaches the distal end of the appendage, it is released into a wider antennal hemocoel and it flows in the downward direction as it returns to the head. A, anterior; M, medial (toward the dorsal midline of the mosquito). This panel is modified from our earlier paper on the antennal APOs of mosquitoes (Boppana and Hillyer, 2014). (B–E) Graphs comparing hemolymph velocity (B,C) and maximum acceleration (D,E) when hemolymph is traveling in the upward (B,D) and downward (C,E) directions. Mosquitoes were treated with a vehicle control or with CCAP, FMRFamide or SALDKNFMRFamide. *P*-values result from ANOVA and Tukey *post hoc* tests (n=45, 45, 51 and 48 for vehicle control, CCAP, FMRFamide and SALDKNFMRFamide, respectively, in both upward and downward directions).

universal. Depending on the N-terminal sequence of the peptide, and the insect being tested, some FLPs are cardioacceletatory (Cuthbert and Evans, 1989; Robb and Evans, 1990; Duve et al., 1993; Duttlinger et al., 2003), some are cardiodeceleratory (Cuthbert and Evans, 1989; Robb and Evans, 1990; Nichols et al., 1999; Lee et al., 2012), some have complex effects that are dependent on the presence of other molecules (Nichols, 2006), and yet others have no effect on heart physiology (Duve et al., 1993; Nichols et al., 1999; Nichols, 2006). In the present study, we confirm the cardioacceleratory activity of SALDKNFMRFamide and FMRFamide, and for the first time show in a holometabolous insect that FLPs accelerate the antennal APOs. The 14–21% increase in the contraction rate of the antennal APOs observed in the present study is in general agreement with the 0–140% change induced by FLPs on the antennal hearts of the cockroach *Periplaneta americana* (Hertel and Penzlin, 1992; Predel et al., 2004). However, the approach used in the present study diverged from that of previous studies, as we performed our analyses in intact insects, thus maintaining their normal hemocoel pressure and hemolymph components, whereas earlier studies were conducted on dissected specimens that had been immersed in buffer solutions. Furthermore, the basal antennal APO contraction rate of mosquitoes, at approximately 1 Hz, is twice the rate of the antennal APO of dissected cockroaches (Hertel et al., 1985).

Crustacean cardioactive peptide is a cyclic amidated nonapeptide that is produced in a broad range of arthropods, including all sequenced insect lineages. This neuropeptide was discovered in the shore crab, *Carcinus maenas*, where it was shown to have a potent cardioacceleratory effect on semi-isolated heart preparations (Stangier et al., 1987). In insects, CCAP functions in ecdysis, hormone release and the contraction of visceral organs (Veelaert et al., 1997; Donini et al., 2001; Ewer and Reynolds, 2002; Sakai et al., 2004; Arakane et al., 2008; Lahr et al., 2012), and more relevant to the present study, CCAP is cardioacceleratory in both holometabolous and hemimetabolous insects (Furuya et al., 1993; Lehman et al., 1993; Dulcis et al., 2005; Wasielewski and

Skonieczna, 2008; da Silva et al., 2011; Estevez-Lao et al., 2013). In mosquitoes, CCAP increases the heart rate as well as the velocity of hemolymph as it travels through the dorsal vessel (Estevez-Lao et al., 2013). Here, we show for the first time that CCAP accelerates the contraction rate of a circulatory organ other than the dorsal vessel, and specifically, we show that this neuropeptide increases the contraction rate of the antennal APOs of mosquitoes by approximately 13%.

A unique feature of this study is that it shows that neuropeptides increase not only the contraction rate of the antennal APOs, but also the velocity of hemolymph in the antennal space. Generally, the increase in hemolymph velocity was higher in the upward direction when compared with the increase in the downward direction, and regardless of the treatment, the velocity of hemolymph was approximately 3.5 times faster in the upward direction when compared with the downward direction. This reduction in velocity occurs at the distal end of the antennae, when hemolymph transitions from a narrow antennal vessel to a wider antennal hemocoel (Fig. 5A) (Boppana and Hillyer, 2014). Also because of this transition, and because of the increased distance from the contractile pump, the maximum acceleration of hemolymph diminishes after the transition from upward to downward flow. Taken altogether, these data show that these three neuropeptides increase the velocity and maximum acceleration of hemolymph in the antennal space, suggesting that these contractions are not only more frequent but also more forceful.

The antennae of mosquitoes are involved in critical physiological processes. The Johnston's organs that are located within the pedicel of each antenna, for example, detect auditory cues and influence courtship and mating behaviors (Cator et al., 2009). Moreover, antennal receptor neurons located within the sensilla of the flagellum detect odorant and thermal cues, which modulate host-seeking (i.e. blood feeding) and oviposition (Wang et al., 2009; Rinker et al., 2013; Suh et al., 2016). Because of the critical role they play in survival and reproduction, mosquitoes must ensure that molecules required for proper antennal functioning are efficiently transported into and out of these appendages. This transport is driven by the antennal auxiliary hearts. Identifying the cellular sources of the neuropeptides that modulate the contraction dynamics of the antennal APOs of mosquitoes was not a focus of this study. However, the antennal APOs of insects, or cells associated with the antennal APO of insects, are neurohemal organs, and among the peptides present within these neurons are proctolin and FLPs (Beattie, 1976; Pass et al., 1988a,b; Woodhead et al., 1992; Mobius and Penzlin, 1993; Predel et al., 1999, 2004; Predel, 2001; Siju et al., 2014). In mosquitoes, transcription of CCAP and SALDKNFMRFamide is highest in the head when compared with the thorax and the abdomen (Estevez-Lao et al., 2013; Hillyer et al., 2014). Within the head of *A. gambiae*, CCAP is produced in the protocerebrum and the subesophageal ganglion (the antennae were not analyzed), and FLPs have been identified in the antennal lobe of *A. aegypti* (Estevez-Lao et al., 2013; Siju et al., 2014). Thus, the antennal APOs should have ample access to these myotropic factors, and perhaps physiological changes related to courtship, mating, host-seeking and oviposition are accompanied by the changes in the production and localized release of these neuropeptides.

Finally, though the antennal APOs and the heart do not contract in synchrony, CCAP and FLPs accelerate both of these contractile pumps. Other neuropeptides and neurotransmitters, such as allatotropin, glutamate and serotonin, increase the heart rate of insects (Veenstra et al., 1994; Johnson et al., 1997; Dulcis and Levine, 2005; Hillyer et al., 2015; Villalobos-Sambucaro et al., 2015). Future studies should assess whether these and other cardiomodulatory factors also accelerate the contraction rate of the antennal APOs, or the contraction rate of other circulatory structures.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
J.F.H. conceived the study. J.M.S., T.H.J. and J.F.H. designed the experiments. J.M.S. and T.H.J. performed the experiments. J.M.S., T.H.J., S.C.M. and J.F.H. analyzed the data. J.F.H. wrote the manuscript.

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