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Myotropic effects of FMRFamide containing peptides on the heart of the mosquito *Anopheles gambiae*

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ABSTRACT

FMRFamide-like peptides (FLPs) are produced by invertebrate and vertebrate animals, and regulate diverse physiological processes. In insects, several FLPs modulate heart physiology, with some increasing and others decreasing dorsal vessel contraction dynamics. Here, we describe the *FMRFamide* gene structure in the mosquito, *Anopheles gambiae*, quantify the developmental and spatial expression of *FMRFamide* and its putative receptor (*FMRFamideR*), and show that the peptides FMRFamide and SALDKNFMRFamide have complex myotropic properties. RACE sequencing showed that the FMRFamide gene encodes eight putative FLPs and is alternatively spliced. Of the eight FLPs, only one is shared by *A. gambiae*, *Aedes aegypti* and *Culex quinquefasciatus*: SALDKNFMRFamide. Quantitative PCR showed that peak expression of *FMRFamide* and *FMRFamideR* occurs in second instar larvae and around eclosion. In adults, *FMRFamide* is primarily transcribed in the head and thorax, and *FMRFamideR* is primarily transcribed in the thorax. Intravital video imaging of mosquitoes injected FMRFamide and SALDKNFMRFamide revealed that at low doses these peptides increase heart contraction rates. At high doses, however, these peptides decrease heart contraction rates and alter the proportional directionality of heart contractions. Taken altogether, these data describe the *FMRFamide* gene in *A. gambiae*, and show that FLPs are complex modulators of mosquito circulatory physiology.

1. Introduction

Insects employ an open circulatory system to transport nutrients, hormones, wastes and immune molecules to their target sites (Chapman et al., 2013; King and Hillyer, 2012; Klowden, 2013). This circulatory system is composed of a fluid medium called hemolymph, an open body cavity called the hemocoel, and a series of pumps that drive hemolymph propulsion. The primary pump is the dorsal vessel, which is a muscular tube that extends the length of the insect and is divided into a thoracic aorta and an abdominal heart (Glenn et al., 2010; Jones, 1977; Leodido et al., 2013). Hemolymph is propelled through the dorsal vessel by wave like contractions of heart muscle. In many insect orders these contractions periodically alternate between propagating in anterograde (toward the head) and retrograde (toward the posterior abdomen) directions, which leads to the release of hemolymph into the distal ends of the hemocoel (Andereck et al., 2010; Gerould, 1933; Glenn et al., 2010; Wasserthal, 2007).

The rate and mode of insect heart contractions varies between species. The heart of the stick insect *Baculum extradentatum* (order: Phasmatodea) contracts only in the anterograde direction and does so at approximately 0.2 Hz (Ejaz and Lange, 2008). In contrast, the heart of the mosquito *Anopheles gambiae*, the fruit fly *Drosophila melanogaster* and the hoverfly *Episyrphus balteatus* (all in order: Diptera) experiences heartbeat directional reversals and contracts at approximately 2 Hz, 4 Hz and 7 Hz, respectively (Estevez-Lao et al., 2013; Slama, 2012; Wasserthal, 2007). Regardless of the contraction rate or the presence of heartbeat directional reversals, it is generally accepted that although the heart contracts myogenically, several neurohormones and neurotransmitters influence contraction dynamics (Chapman et al., 2013; Klowden, 2013). For example, crustacean cardioactive peptide (CCAP) increases heart rates (Chen and Hillyer, 2013; Dulcis et al., 2005; Ejaz and Lange, 2008; Estevez-Lao et al., 2013), neuropeptide F regulates the slow phase of cardiac activity (Setzu et al., 2012), corazonin increases heart rates in some insects but not others (Hillyer et al., 2012; Veenstra, 1989), and serotonin and glutamate have cardioacceleratory properties (Dulcis and Levine, 2005; Johnson et al., 1997).

Besides these myotropic factors, a group of neuropeptides that influences insect cardiac activity is the FMRFamide-like peptides (FLPs), often referred to as the FMRFamide-related peptides

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(FaRPs) (Merte and Nichols, 2002; Orchard et al., 2001; Walker et al., 2009). FLPs, defined by the presence of a conserved C-terminal RFamide and a unique N-terminal sequence, were initially discovered in the clam *Macrocallista nimbosa* because of the cardioacceleratory activity of the tetrapeptide FMRFamide (Price and Greenberg, 1977). Since, FLPs have been identified in a broad range of invertebrates and vertebrates and shown to modulate numerous physiological processes (Walker et al., 2009). As pertains to cardiac physiology, different insect FLPs are known to (1) be cardioacceleratory (Cuthbert and Evans, 1989; Duttlinger et al., 2003; Duve et al., 1993; Robb and Evans, 1990), (2) be cardiodeceleratory (Cuthbert and Evans, 1989; Lee et al., 2012; Nichols et al., 1999; Robb and Evans, 1990), (3) have complex effects that are dependent on the presence of other molecules (Nichols, 2006), and (4) have no effect on heart physiology (Duve et al., 1993; Nichols, 2006; Nichols et al., 1999). The mechanism by which different FLPs modulate heart physiology remains unknown, but one possibility is that, for FMRFamide containing peptides, the C-terminal tetrapeptide binds the receptor whereas the variable N-terminal sequence drives the physiological activity of each peptide variant (Cazzamali and Grimmelikhuijzen, 2002; Maynard et al., 2013).

In mosquitoes, FMRFamide immunoreactivity has been detected in endocrine cells of the midgut, and in nervous tissue of the supraesophageal and subesophageal ganglia, the ventral nerve cord, the antennal lobe and several visceral organs (Brown et al., 1986; Brown and Lea, 1988; Moffett and Moffett, 2005; Siju et al., 2013). Furthermore, several RF-containing peptides have been empirically identified by mass spectrometric profiling of mosquito tissues (Predel et al., 2010). Injection of mosquito larvae with FMRFamide increases heart rates (Duttlinger et al., 2003), but the effect of FMRFamide or any other FLP on adult heart physiology remains unknown. Here, we describe the structure of the *FMRFamide* gene in the malaria mosquito *A. gambiae*, assess the developmental expression of *FMRFamide* and the putative FMRFamide receptor, and show that the peptides FMRFamide and SALDKNFMRFamide have deceleratory and acceleratory effects on adult heart physiology.

2. Materials and methods

2.1. Mosquito rearing and maintenance

A. gambiae (Diptera: Culicidae), G3 strain, were reared and maintained in an environmental chamber at 27 °C and 75% humidity as described (Glenn et al., 2010). Briefly, eggs were hatched in water and larvae were fed a combination of koi fish food and baker's yeast. Pupae were transferred to 1.5 L containers, and after emergence, adults were fed a 10% sucrose solution ad libitum. All experiments were carried out on 4-day-old adult female mosquitoes.

2.2. cDNA synthesis, PCR, sequencing and rapid amplification of cDNA ends

cDNA synthesis, PCR, sequencing and rapid amplification of cDNA ends (RACE) were all performed essentially as described (Estevez-Lao et al., 2013). For cDNA synthesis, RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), re-purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and treated with RQ1 RNAse-free DNAse (Promega, Madison, WI, USA). cDNA was then synthetized using an Oligo(dt)$_{20}$ primer and the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). RNA was subsequently degraded using RNase H.

To sequence *FMRFamide*, the central region of *A. gambiae FMRFamide* was first amplified from cDNA using gene-specific primers and high fidelity/high specificity Accuprime Pfx SuperMix (Invitrogen). Amplicons were purified using the PureLink PCR Purification Kit (Invitrogen) and cloned using Invitrogen's TOPO TA Cloning Kit for Sequencing. The plasmids were then isolated using Qiagen's Plasmid Mini Kit and their inserts were sequenced using BigDye Terminator v3.1 chemistry (Applied Biosystems, Foster City, CA, USA) at Vanderbilt University's DNA sequencing facility. The sequencing trace files were analyzed using 4Peaks software (Mek&Tosj, Amsterdam, The Netherlands).

The 5′ and 3′ terminal ends of *FMRFamide* were sequenced using 5′/3′ RACE libraries constructed using Invitrogen's GeneRacer kit. These libraries were previously used to determine the gene structures of other neuropeptide and immunity genes (Estevez-Lao et al., 2013; Estevez-Lao and Hillyer, 2014; Hillyer et al., 2012). For both 5′ and 3′ RACE, the transcript was amplified by PCR using a gene-specific primer and a GeneRacer primer. The PCR products were purified and used as template in a second PCR reaction that contained a nested gene-specific primer and a nested GeneRacer primer. The amplicons were then separated by agarose gel electrophoresis, excised, purified, cloned, sequenced and analyzed as described above. For a list of primers used in this study see Table S1 in Supplementary file 1.

Nucleotide sequences were manually assembled after alignment in Serial Cloner (http://serialbasics.free.fr/Serial_Cloner.html), and the gene structure was graphically visualized using Artemis software (Wellcome Trust Sanger Institute, Cambridge, UK). The predicted protein mass of FMRFamide was calculated using the Compute pI/Mw tool in ExPASy (http://web.expasy.org/compute_pi/), and the location of the signal peptide was predicted using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). TBLASTN searches were performed on the GenBank nucleotide database in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and multiple sequence alignments were performed by Multiple Alignment using Fast Fourier Transform (MAFFT; http://mafft.cbrc.jp/alignment/server/).

2.3. Developmental and spatial gene expression analyses

Gene expression of *FMRFamide* and the putative FMRFamide receptor (*FMRFamideR*) was quantified as described for *CCAP* (Estevez-Lao et al., 2013). For developmental analyses, cDNA was synthetized from RNA purified from eggs (containing developing 1st instar larvae), 2nd instar larvae, 3rd instar larvae, 4th instar larvae, pupae (callow or black), or adults (1, 5 or 10 days old). Relative *FMRFamide* and *FMRFamideR* transcript levels were measured by real-time quantitative PCR (qPCR) using SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 Real-Time PCR system. *RPS7* was used as the reference, as this gene has been validated in identical (Estevez-Lao et al., 2013) and similar (Coggins et al., 2012) studies. Relative quantification of *FMRFamide* and *FMRFamideR* mRNA levels was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The graphed output displays the average fold-change in mRNA levels relative to eggs.

For spatial expression analyses, cDNA was synthetized from RNA purified from whole bodies, heads, thoraces or abdomens from adult female mosquitoes at 4 days post-eclosion. Transcript levels were analyzed as above, with the graphed output displaying the average mRNA fold-difference relative to whole bodies. For both developmental and spatial expression studies, six and five biological replicates were performed for *FMRFamide* and *FMRFamideR*, respectively, and each was analyzed in duplicate.

2.4. Measurement of heart physiology following neuropeptide injection

FMRFamide and SALDKNFMRFamide were purchased from Bachem Americas Inc. (Torrance, CA, USA). The peptides were

reconstituted in PBS at a concentration of 1×10^{-2} M, aliquoted, and frozen. Immediately prior to each experiment, the peptide solutions were thawed and diluted in PBS to the concentrations to be used.

For FMRFamide experiments, mosquitoes were cold-anesthetized for 60 s, placed dorsal side up on Sylgard 184 silicone elastomer plates (Dow Corning Corp., Midland, MI, USA), and restrained using a non-invasive method previously described and pictured (Andereck et al., 2010). After each mosquito acclimated to room temperature, a 60 s intravital video was recorded by imaging through the dorsal abdominal cuticle using bright field trans illumination on a Nikon SMZ1500 stereo microscope (Nikon Corp., Tokyo, Japan) connected to an ORCA-Flash 2.8 high speed monochrome CMOS camera (Hamamatsu Photonics, Hamamatsu City, Japan) and Nikon Advanced Research NIS-Elements software. Each mosquito was then injected through the thoracic anepisternal cleft with 0.1–0.2 µl of phosphate buffered saline (pH 7.0; PBS) or FMRFamide in PBS (injection concentrations ranging from 1×10^{-2} M to 1×10^{-8} M). Then, additional 60 s recordings were taken at 2, 10 and 30 min post-injection. Thus, serial videos were acquired for each mosquito, yielding basal heart physiology and the effect of peptide exposure at 2, 10 and 30 min post-treatment.

For SALDKNFMRFamide experiments, mosquitoes were anesthetized, restrained and imaged as above. A video of each mosquito was acquired prior to injection and at 10 min post-injection (injection concentrations ranging from 1×10^{-2} M to 1×10^{-8} M). This approach also yielded paired data: basal heart physiology and the effect of peptide exposure at 10 min post-treatment.

By visualizing the direction and frequency of heart contractions, the following parameters were measured: (1) total, anterograde and retrograde contraction rates (Hertz; Hz; contractions/s), (2) frequency of heartbeat directional reversals (reversals from contracting anterograde to contracting retrograde and vice versa), (3) percentage of contractions propagating in anterograde and retrograde directions, (4) percentage of time the heart contracts in anterograde and retrograde directions. For videos of contracting hearts, see our previously published work (Andereck et al., 2010; Estevez-Lao et al., 2013; Glenn et al., 2010; Hillyer et al., 2012).

2.5. Statistical analyses of heart physiological data

Physiological experiments were conducted such that they yielded paired data: measurements before treatment and after treatment. The paired data were analyzed using repeated measures two-way ANOVA, with the *P*-value relevant to this study assessing whether there is a significant dose-dependent effect of peptide injection on heart physiology (interaction). In addition, Sidak's post hoc tests were used to compare the pre-injection values of a given group (PBS or a specific peptide dose) with the post-injection values of that same group. All statistical tests where conducted in GraphPad Prism 6 (La Jolla, CA), and differences were deemed significant at $P < 0.05$.

3. Results

3.1. A. gambiae FMRFamide gene structure

The *A. gambiae FMRFamide* gene was initially identified during a genome screen that aimed to catalog putative neuropeptides and peptide hormones (Riehle et al., 2002). *FMRFamide* was predicted to encode up to eight FLPs, with one of these peptides containing an FMRF sequence: SALDKNFMRFamide. We used this FLP as the query sequence in a BLASTP search of the AgamP3 assembly of the *A. gambiae* genomic sequence (available in www.vectorbase.org). Results of this analysis revealed a single copy gene, AGAP005518, which is located in chromosome 2 L. To determine the *A. gambiae FMRFamide* transcript structure, the central portion of the *FMRFamide* mRNA was amplified and sequenced using cDNA synthetized from adult female mosquitoes and primers specific to AGAP005518. Then, the 5′ and 3′ ends of *FMRFamide* were sequenced using 5′ and 3′ RACE libraries, and gene-specific and vector-specific primers.

Analysis of the assembled *FMRFamide* sequences revealed that this gene is alternatively spliced (Fig. 1). The first splice variant (Genbank ID: KJ583231) is 1916 base pairs (bp) in length, and is composed of a 340 bp 5′ untranslated region (UTR), a 993 bp open reading frame (ORF), and a 583 bp 3′ UTR. Alignment of the first splice variant to the *A. gambiae* genomic sequence revealed that it is composed of two exons that are 254 and 1662 bp in length, which are separated by a 24,017 bp intron. Conceptual translation of the mRNA predicts a 330 amino acid (aa) protein precursor with a predicted mass of 36.2 kDa. The ORF is predicted to encode a 21 aa signal peptide and, as previously predicted by Riehle et al. (Riehle et al., 2002), eight FLPs: SALDKNFMRFamide, TDKTVARQT RANLMRFamide, PDRNFLRFamide, DSPKNLMRFamide, STGSG YMRFamide, AGNLMRFamide (encoded twice), and AARAGPNL MRFamide (Table 1). The sequence of all putative FLPs is preceded by one or more basic residues (R, RR, RRR or KRR), and succeeded by a glycine and one or more basic residues (GR or GKRR). The basic amino acids and the glycines serve as proteolytic cleavage

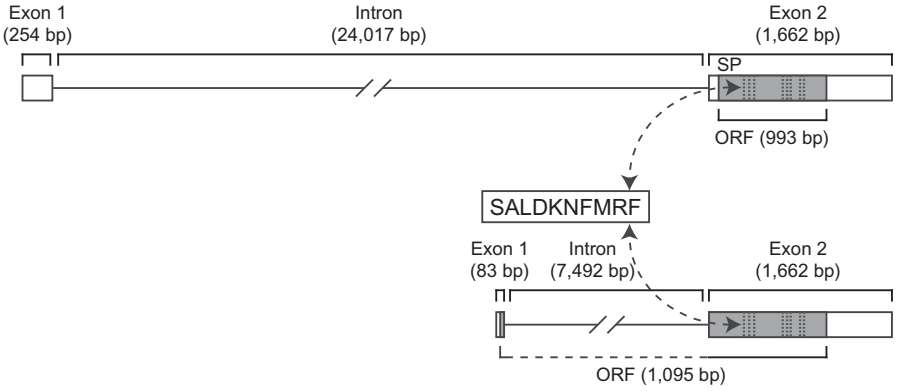

Fig. 1. Gene structure of *Anopheles gambiae FMRFamide*, showing both splice variants. The open reading frames (ORFs) are the grey boxes, the untranslated regions (UTRs) are the white boxes, and the introns are the horizontal lines. The predicted signal peptide is marked SP, and the locations where the FLPs are encoded are marked within the ORF with vertical dotted lines. From left to right the FLPs are: SALDKNFMRFamide, TDKTVARQTRANLMRFamide, PDRNFLRFamide, DSPKNLMRFamide, STGSGYMRFamide, AGNLMRFamide, AARAGPNLMRFamide, and AGNLMRFamide. GenBank accession numbers are: Splice variant 1 (top), KJ583231; Splice variant 2 (bottom), KJ583232. For an alignment of dipteran FMRFamide sequences see Fig. S1 in Supplementary file 1.

Table 1
Putative peptides encoded by *Anopheles gambiae FMRFamide*, including their positions in the mRNA (nucleotide, bp) and protein (amino acid, aa) sequences.

	Peptide[a]	mRNA position (bp)		Protein position (aa)	
		Variant 1[b]	Variant 2[b]	Variant 1[b]	Variant 2[b]
1	SALDKNFMRFamide	575–604	404–433	79–88	113–122
2	TDKTVARQTRANLMRFamide	611–658	440–487	91–106	125–140
3	PDRNFLRFamide	665–688	494–517	109–116	143–150
4	DSPKNLMRFamide	923–949	752–778	195–203	229–237
5	STGSGYMRFamide	962–988	791–817	208–216	242–250
6	AGNLMRFamide	995–1015	824–844	219–225	253–259
7	AARAGPNLMRFamide	1076–1108	905–937	246–256	280–290
8	AGNLMRFamide	1115–1135	944–964	259–265	293–299

[a] SALDKNFMRFamide is the only FLP conserved across the mosquito lineage, and is the only *A. gambiae* FLP that contains a C-terminal FMRFamide.
[b] GenBank accession numbers are: Splice variant 1, KJ583231; Splice variant 2, KJ583232.

signals and C-terminal amidation signals, respectively (Eipper and Mains, 1988; Loh and Gainer, 1983).

The second splice variant (Genbank ID: KJ583232) is 1745 bp in length, and is composed of a 67 bp 5′ UTR, a 1095 bp ORF, and a 583 bp 3′ UTR. Alignment of the second splice variant to the *A. gambiae* genomic sequence revealed that it is composed of two exons that are 83 and 1662 bp in length, which are separated by a 7492 bp intron. The second exon is identical in both splice variants. Conceptual translation of the mRNA predicts a 364 aa protein precursor with a predicted mass of 39.8 kDa. The ORF is predicted to encode the eight FLPs also encoded in the first splice variant but is not predicted to encode a signal peptide (Table 1). However, the entire predicted ORF of splice variant 1 is contained within the predicted ORF of splice variant 2, and both ORFs occur in frame. Alternative translation start sites are common in vertebrates and invertebrates (Bazykin and Kochetov, 2011), so perhaps the protein precursors are the same for both splice variants.

A TBLASTN search of the GenBank nucleotide database in NCBI using the conceptual translation of splice variant 1 identified the *FMRFamide* genes in the mosquitoes (order: Diptera, suborder: Nematocera) *Aedes aegypti* (XM_001663782.1) and *Culex quinquefasciatus* (XM_001841719.1), and in the brachyceran flies (order: Diptera, suborder: Brachycera) *Ceratitis capitata* (XM_004526012.1), *Musca domestica* (AB214648.1) and *D. melanogaster* (NM_078945.3). Sequence alignment by MAFFT revealed little perfect conservation between these dipteran sequences, except for the locations surrounding and containing the RF residues (Fig. S1 in Supplementary file 1). A search for the eight *A. gambiae* FLPs in the dipteran sequences showed that none are conserved across all taxa. Only one is conserved across the mosquito lineage (SALDKNFMRFamide), and another is present in *A. gambiae* and *A. aegypti* but not in *C. quinquefasciatus* (DSPKNLMRFamide). Both of these peptides have been empirically identified by mass spectrometric profiling of *A. aegypti* tissues (Predel et al., 2010).

3.2. Developmental expression of FMRFamide and FMRFamideR

To determine the developmental expression of *FMRFamide* and the putative FMRFamide receptor (*FMRFamideR*), cDNA was synthetized from the whole bodies of mosquitoes at all developmental stages, and mRNA levels were subsequently measured by quantitative PCR. Primers for *FMRFamide* were designed using the sequences determined by RACE (Fig. 1). *FMRFamideR* (AGAP001862) was identified by sequence homology to the bioactive FMRFamide receptor in *D. melanogaster* (Cazzamali and Grimmelikhuijzen, 2002; Meeusen et al., 2002), and primers were designed using the bioinformatic prediction of the gene (Duttlinger et al., 2003; Vogel et al., 2013).

Analysis of the developmental data revealed that expression of *FMRFamide* and *FMRFamideR* shows a bimodal distribution, with expression peaks occurring in second instar larvae and around eclosion (Fig. 2). When relative expression of the two genes was compared around the time of eclosion, mRNA levels of *FMRFamideR* peaked first. Specifically, expression of *FMRFamideR* peaked in pupae whereas expression of *FMRFamide* peaked in 1-day-old adults and remained elevated till at least 10 days post-eclosion. For both *FMRFamide* and *FMRFamideR* the lowest levels of expression were seen in eggs. Finally, relative to eggs, developmental regulation was significantly higher for *FMRFamide* when compared to *FMRFamideR* (113- vs. 23-fold difference).

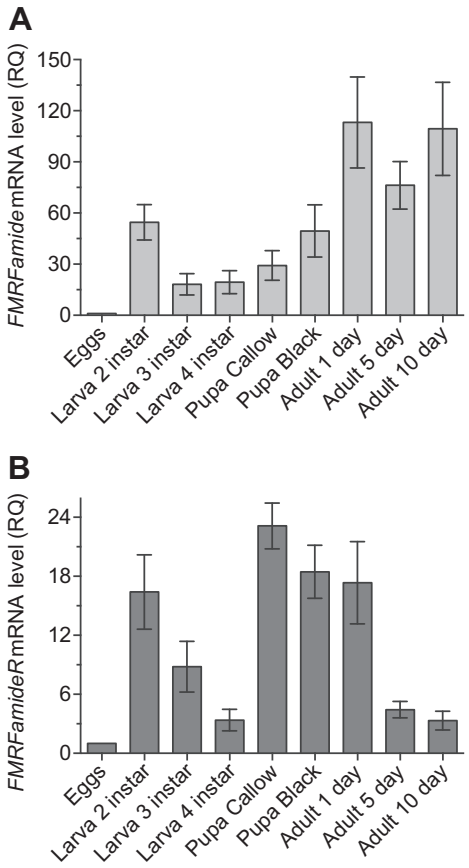

Fig. 2. Developmental expression of *Anopheles gambiae FMRFamide* and the putative FMRFamide receptor (*FMRFamideR*). Quantitative RT-PCR analysis of the transcription of *FMRFamide* (A) and *FMRFamideR* (B) in eggs, 2nd through 4th instar larvae, callow (early) and black (late) pupae, and adults at 1, 5 and 10 days after eclosion. The graph displays the average fold-difference in mRNA levels relative to eggs (relative quantification; RQ), using *RPS7* as the reference gene. Whiskers denote the standard error of the mean.

3.3. Spatial expression of FMRFamide and FMRFamideR

To determine the spatial expression of *FMRFamide* and *FMRFamideR*, cDNA was synthetized from the whole bodies, heads, thoraces and abdomens of 4-day-old adult female mosquitoes, and mRNA levels were measured by quantitative PCR. Analyses of these data showed that *FMRFamide* is primarily transcribed in the head and thorax whereas *FMRFamideR* is primarily transcribed in the thorax (Fig. 3). Levels of *FMRFamide* and *FMRFamideR* mRNA were barely detectable in the abdomen.

3.4. Basal heart physiology

Analysis of videos taken prior to any treatment ($n = 175$) revealed that the mosquito heart contracts at a basal rate of 1.93 Hz (+/− 0.37 S.D.), with contractions propagating in the anterograde and retrograde directions at 1.90 Hz (+/− 0.41 S.D.) and 1.97 Hz (+/− 0.36 S.D.), respectively. An average of 65% (+/− 14 S.D.) of contractions propagate in the anterograde direction and 35% (+/− 14 S.D.) of contractions propagate in the retrograde direction. Similarly, the heart spends 66% (+/− 13 S.D.) and 34% (+/− 14 S.D.) of the time contracting anterograde and retrograde, respectively. Finally, the heart reverses contraction direction an average of 11.6 times per minute (+/− 5.3 S.D.). These data are in agreement with our previously published work (Chen and Hillyer, 2013; Estevez-Lao et al., 2013; Glenn et al., 2010; Hillyer et al., 2012).

3.5. Myotropic activity of FMRFamide

Because FLPs were first discovered in the clam *M. nimbosa* because of the cardioacceleratory activity of FMRFamide (Price and Greenberg, 1977), we tested whether this tetrapeptide affects mosquito heart physiology. Injection of PBS resulted in a short-lived increase in mosquito heart rates. At 2 min following injection with PBS, the total, anterograde and retrograde heart rates had increased by 15%, 19% and 6%, respectively. However, by 10 min post-treatment heart rates had returned to pre-injection levels. The process of injection had no effect on the percentage of contractions or the percentage of the time the heart contracted in a given direction, but decreased the frequency of heart beat directional reversals.

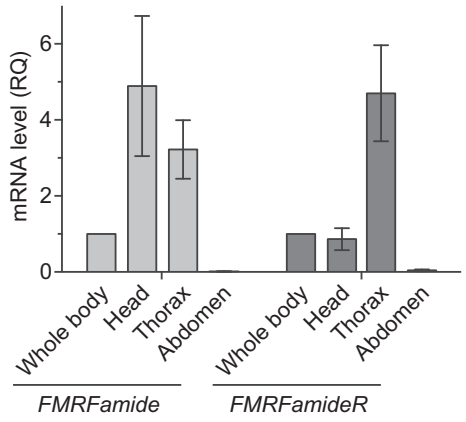

Fig. 3. *Anopheles gambiae FMRFamide* and *FMRFamideR* expression in different body segments. Quantitative RT-PCR analysis of *FMRFamide* and *FMRFamideR* mRNA levels in the whole bodies, heads, thoraces and abdomens of adult, female mosquitoes at 4 days of age. The graph displays the average fold-difference in mRNA levels relative to whole bodies (relative quantification; RQ), using *RPS7* as the reference gene. Whiskers denote the standard error of the mean.

Treatment with FMRFamide affected heart contraction rates in a dose dependent manner (Figs. 4A–C, 5A–C). Whereas the effect of FMRFamide at 1×10^{-8} M and 1×10^{-4} M was similar to the effect of PBS (increased rates at 2 min post-injection but recovery by 10 min), injection with FMRFamide at 1×10^{-6} M resulted in increased heart rates that persisted until at least 30 min post-treatment (22%, 27% and 13% increases in total, anterograde and retrograde rates, respectively). However, the most striking (and opposite) effect occurred after treatment with FMRFamide at 1×10^{-2} M. This treatment induced a >40% decrease in total, anterograde and retrograde heart rates by 2 and 10 min post-treatment. By 30 min post-treatment heart rates had returned to basal levels.

Treatment with FMRFamide also had a dose-dependent effect on the proportional directionality of heart contractions (Figs. 4D–E, G–H, 5E–F). The most significant and marked effect was that injection with FMRFamide at 1×10^{-2} M resulted in a greater than twofold increase in the percentage of contractions that propagated in the retrograde direction and the percentage of time the heart contracted in the retrograde direction. This effect was seen at 2 and 10 min post-treatment, but by 30 min post-treatment heart physiology had returned to basal levels.

Treatment with FMRFamide did not have a dose-dependent effect on the frequency of heartbeat directional reversals (Figs. 4F, 5D). The process of injection led to a decrease in the frequency of heartbeat directional reversals, but interestingly, the lowest decrease in the frequency of heartbeat directional reversal was observed after treatment with FMRFamide at 1×10^{-2} M. Taken altogether, these data show that low concentrations of FMRFamide accelerate heart rates whereas high concentration of FMRFamide (1) decelerate heart rates and (2) alter the proportional directionality of heart contractions. For an expansion of the data in Fig. 5, where each dose is graphed independently, see Figs. S2–S7 in Supplementary file 1. To further visualize the dose dependent effect of FMRFamide on mosquito heart physiology, see Fig. S8 in Supplementary file 1.

3.6. Myotropic activity of SALDKNFMRFamide

Given that the tetrapeptide FMRFamide affected heart physiology in a bimodal and dose-dependent manner, we then tested whether one of the peptides encoded in *A. gambiae FMRFamide* has similar properties. Specifically, we tested the effect of SALDKNFMRFamide on heart physiology at 10 min post-treatment. This peptide was selected because it is the only FLP that is encoded in the genomes of *A. gambiae*, *A. aegypti* and *C. quinquefasciatus*, and because it is the only *A. gambiae* FLP that contains an FMRFamide sequence (Fig. 1, Table 1, and Fig. S1 in Supplementary file 1).

In this experiment, injection with PBS resulted in a modest, 9% increase in heart rates at 10 min post-injection, and the process of injection had no effect on the percentage of contractions or the percentage of time the heart contracted in a given direction. However, injection decreased the frequency of heartbeat directional reversals.

Analysis of the SALDKNFMRFamide heart rate data revealed dose-dependent effects that were similar to those observed after treatment with FMRFamide (Figs. 6A–C, 7A–C). First, low doses of SALDKNFMRFamide induced increases in mosquito heart rates whereas high doses induced decreases in heart rates. The effect was most pronounced for higher doses of SALDKNFMRFamide: 1×10^{-6} M and 1×10^{-4} M increased the total heart rate by 16% and 18%, respectively, whereas 1×10^{-2} M decreased the total heart rate by 29%.

Also similar to what was observed for FMRFamide was that treatment with SALDKNFMRFamide had a dose-dependent effect on the proportional directionality of heart contractions (Figs. 6D–E, G–H, 7E–F). Again, the most pronounced effect was seen for

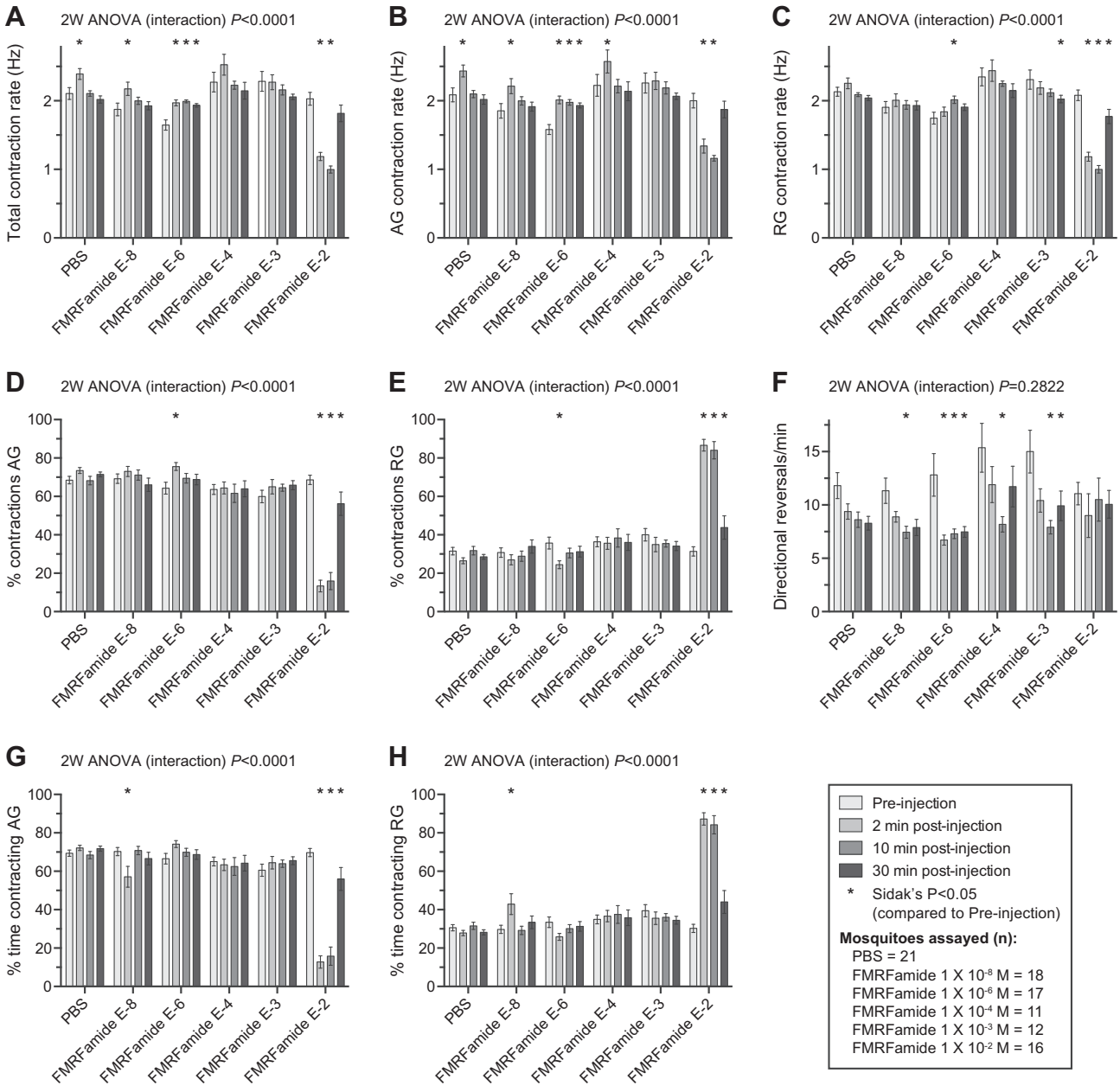

Fig. 4. Effect of FMRFamide injection on mosquito heart physiology. Heart physiological recordings were acquired before (pre) and at 2, 10 and 30 min post-injection with FMRFamide doses ranging from 0 M (PBS) to 1×10^{-2} M. Columns heights mark the mean and the whiskers denote the standard error of the mean. The *P*-value at the top of each graph compares the pre- and post-treatment values using repeated measures two-way ANOVA, and shows whether there is an interaction between FMRFamide dose and changes in heart physiology. An asterisk above a column means that there is a statistical difference (Sidak's post hoc test $P < 0.05$) between that column and the pre-injection column of that group (e.g., pre-injection of PBS vs. 2 min after injection of PBS). The heart parameters measured were the total, anterograde (AG) and retrograde (RG) contraction rates (A–C), the percentage of contractions propagating in the anterograde and retrograde directions (D, E), the frequency of heartbeat directional reversals (F), and the percentage of time spent contracting in the anterograde and retrograde directions (G, H). Sample sizes and abbreviations are detailed in the box at the bottom right of the figure.

1×10^{-2} M, which increased the percentage of contractions propagating in the retrograde direction and the percentage of time the heart contracted in the retrograde direction. Finally, whereas PBS injection reduced the frequency of heartbeat directional reversals, this effect was attenuated by injection of SALDKNFMRFamide (Figs. 6F, 7D). Taken altogether, these data show that SALDKNFMRFamide has dose-dependent cardioacceleratory and cardiodecelerator properties. SALDKNFMRFamide and FMRFamide have similar cardiomyotropic properties, but the magnitudes of the effects are lower following treatment with SALDKNFMRFamide.

4. Discussion

In this study we determine the gene structure of *A. gambiae FMRFamide*, describe the developmental and spatial expression of *FMRFamide* and *FMRFamideR*, and show that the peptides FMRFamide and SALDKNFMRFamide have complex cardiomyotropic properties. By sequencing *FMRFamide* we found that, similar to *FMRFamide* in other invertebrates, this gene encodes multiple putative FLPs. Also similar to other FLP-encoding genes is that a number of these peptides are encoded consecutively within the

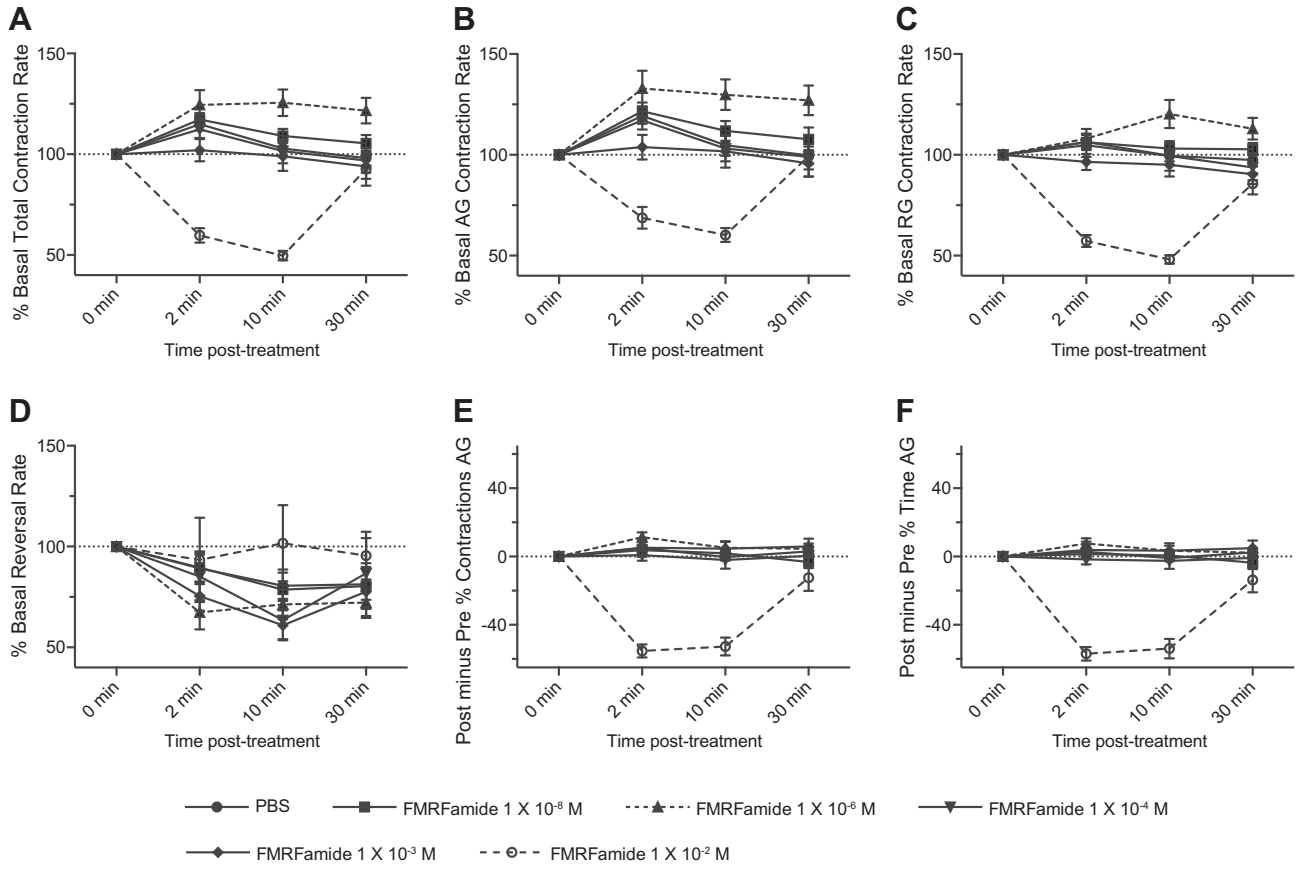

Fig. 5. Time dependent effect of FMRFamide injection on mosquito heart physiology. (A–C) Percent basal total (A), anterograde (B) and retrograde (C) contraction rates. (D) Percent basal frequency of heartbeat directional reversals. For each individual mosquito (A–D), the effect of treatment was calculated by dividing the post-treatment values by the pre-treatment values. (E) Difference in the percentage of contractions propagating in the anterograde direction after and before treatment. (F) Difference in the percentage of time spent contracting in the anterograde direction after and before treatment. For each individual mosquito (E, F), the effect of treatment was calculated by subtracting the value before treatment from the value after treatment (e.g., percent time anterograde after treatment minus percent time anterograde before treatment). For all graphs, the points mark the average and the whiskers denote the standard error of the mean. For an expansion of this graph, where each dose is graphed independently, see Figs. S2–S7 in Supplementary file 1.

ORF. However, different from other dipteran *FMRFamide* genes, including other mosquitoes, is that *A. gambiae FMRFamide* only encodes one FMRF containing peptide. This contrasts with *D. melanogaster FMRFamide*, which encodes five different FMRFamide containing peptides, including one that is encoded five consecutive times within the ORF (Nambu et al., 1988).

A. gambiae FMRFamide is alternatively spliced. Alternative splicing of the *FMRFamide* gene has not been reported in mosquitoes, but has been reported for other invertebrates (Benjamin and Burke, 1994; Cummins et al., 2011). In these mollusks, different splice variants are expressed in different tissues, including the cells that innervate the heart, and encode different complements of peptides (Benjamin and Burke, 1994; Cummins et al., 2011). Thus, it has been proposed that alternative splicing of *FMRFamide* drives the differential distribution of distinct sets of FLPs in defined neuronal networks (Santama and Benjamin, 2000). However, *FMRFamide* alternative splicing in mosquitoes likely has a different effect. Mainly, the two *A. gambiae FMRFamide* splice variants are predicted to encode the same complement of FLPs, making it unclear how different peptide complements would be produced in different tissues. Moreover, the complete ORF of splice variant 1 is encoded in splice variant 2, and both splice variants occur in frame. It is possible that the second splice variant uses an alternative translation site, resulting in both splice variants producing the same protein precursor. The hypothesis of an alternative translation site is supported by the fact that the conceptual translation of splice variant 2 lacks a signal peptide. To our knowledge, this is the only *FMRFamide* conceptual translation that lacks a leader sequence, and given the overabundance of data showing that this peptide is secreted, it is likely that splice variant 2 uses the second predicted methionine as the translation start site, thus yielding a protein precursor that is identical to the protein predicted to be encoded by splice variant 1.

Comparison of the *FMRFamide* and *FMRFamideR* developmental data to the developmental expression scheme of other *A. gambiae* neuropeptides shows some similar patterns. First, the bimodal expression peaks seen here also occur for *A. gambiae CCAP*, corazonin (*CRZ*) and bursicon (*BURS* and *PBURS*) (Estevez-Lao et al., 2013; Hillyer et al., 2012; Honegger et al., 2011), but not Nimrod immunity genes (Estevez-Lao and Hillyer, 2014). Also, as was reported for *CCAP* and *CRZ*, peak expression of the putative FMRFamide receptor precedes peak expression of the FMRFamide peptide (Estevez-Lao et al., 2013; Hillyer et al., 2012).

The primary sites of *FMRFamide* transcription are the head and thorax. This was expected, as FLPs have been detected in the mosquito brain, the supraesophageal ganglion, the subesophageal ganglion and the antennal lobe (Brown and Lea, 1988; Predel et al., 2010; Siju et al., 2013). Furthermore, this finding is in agreement with studies in other insects that have shown by in situ hybridization and mass spectrometry that FLPs are produced and present in the thoracic ganglia (Predel et al., 2004; Taghert, 1999; Wegener et al., 2006). However, this contrasts with studies in mosquitoes

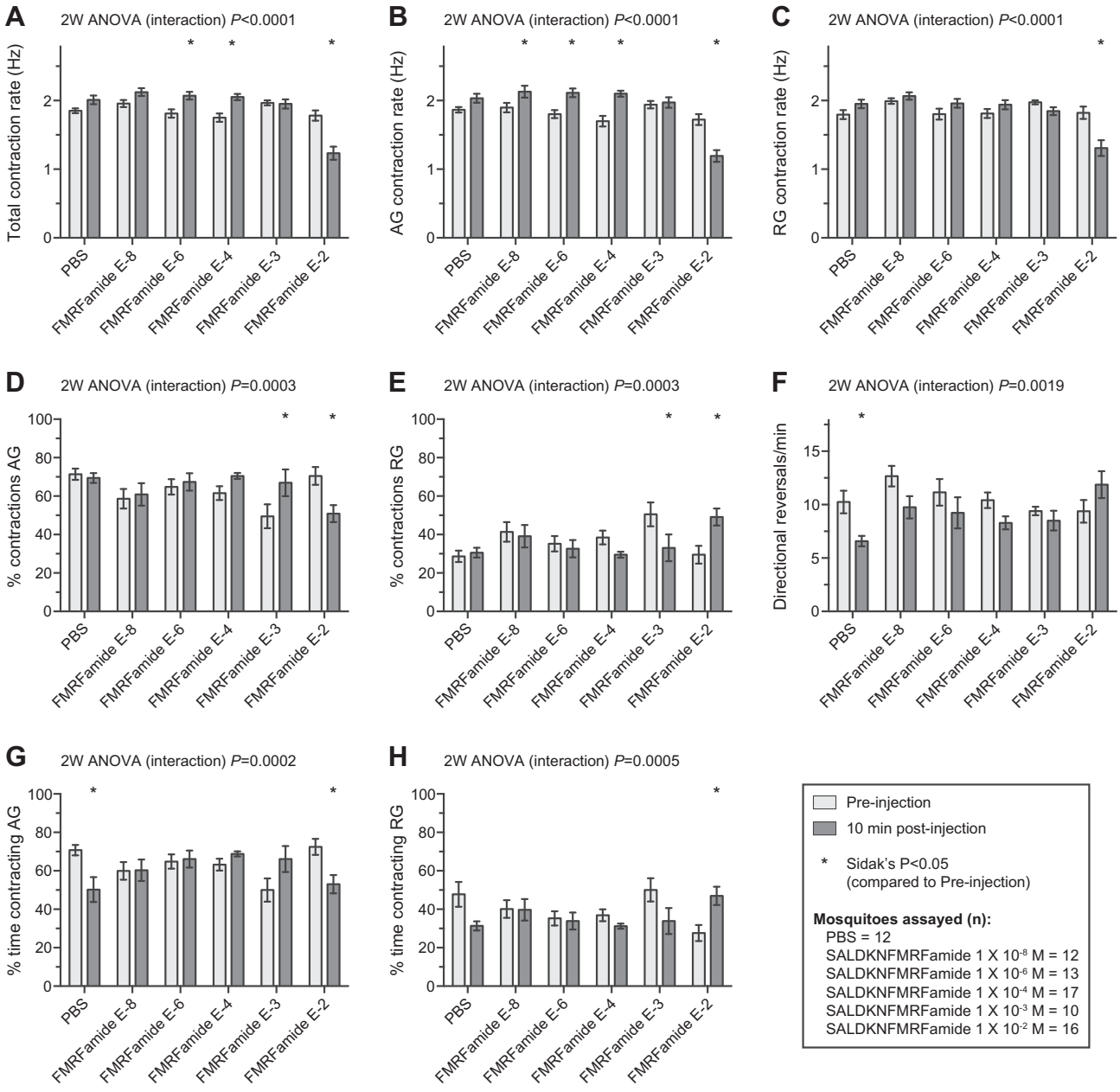

Fig. 6. Effect of SALDKNFMRFamide injection on mosquito heart physiology. Heart physiological recordings were acquired before (pre) and at 10 min post-injection with SALDKNFMRFamide doses ranging from 0 M (PBS) to 1×10^{-2} M. Columns heights mark the mean and the whiskers denote the standard error of the mean. The *P*-value at the top of each graph compares the pre- and post-treatment values using repeated measures two-way ANOVA, and shows whether there is an interaction between SALDKNFMRFamide dose and changes in heart physiology. An asterisk above a column means that there is a statistical difference (Sidak's post hoc test $P < 0.05$) between that column and the pre-injection column of that group. The heart parameters measured were the total, anterograde (AG) and retrograde (RG) contraction rates (A–C), the percentage of contractions propagating in the anterograde and retrograde directions (D, E), the frequency of heartbeat directional reversals (F), and the percentage of time spent contracting in the anterograde and retrograde directions (G, H). Sample sizes and abbreviations are detailed in the box at the bottom right of the figure.

that have shown FLP immunoreactivity in mosquito abdominal ganglia, midguts, and other visceral organs (Brown et al., 1986; Brown and Lea, 1988; Moffett and Moffett, 2005), and studies that have detected FLP immunoreactivity in abdominal neural processes in *D. melanogaster*, *Rhodnius prolixus* (kissing bug), *Schistocerca gregaria* (locust), *Phormia regina* (blow fly), *Tabanus nigrovittatus* (horse fly) and other insects (Haselton et al., 2008; Myers and Evans, 1985; Nichols et al., 1995; Sedra and Lange, 2014; Sevala et al., 1993; Tsang and Orchard, 1991). Finally, of specific interest to the present study, FLP immunoreactivity has also been detected in cells innervating the abdominal heart of *S. gregaria* and *B. extradentatum*, as well as the thoracic aorta of *D. melanogaster* and *R. prolixus* (Calvin and Lange, 2010; Nichols, 2006; Robb and Evans, 1990; Sedra and Lange, 2014; Tsang and Orchard, 1991). Thus, whereas the detection of RFamide immunoreactivity in the abdomen may at first suggest that FLPs are transcribed in the abdomen, there are two primary reasons that explain why this is not the case. First, FLPs are most likely present in the abdomen because of their release into the hemolymph or because of neural processes that extend from the thorax into different tissues of the abdomen (Carroll et al., 1986; Miksys et al., 1997; Myers and Evans, 1985; Sevala et al., 1993). Second, many of the above immunohistochemical studies relied on the detection of RFamide, and thus could have detected many other

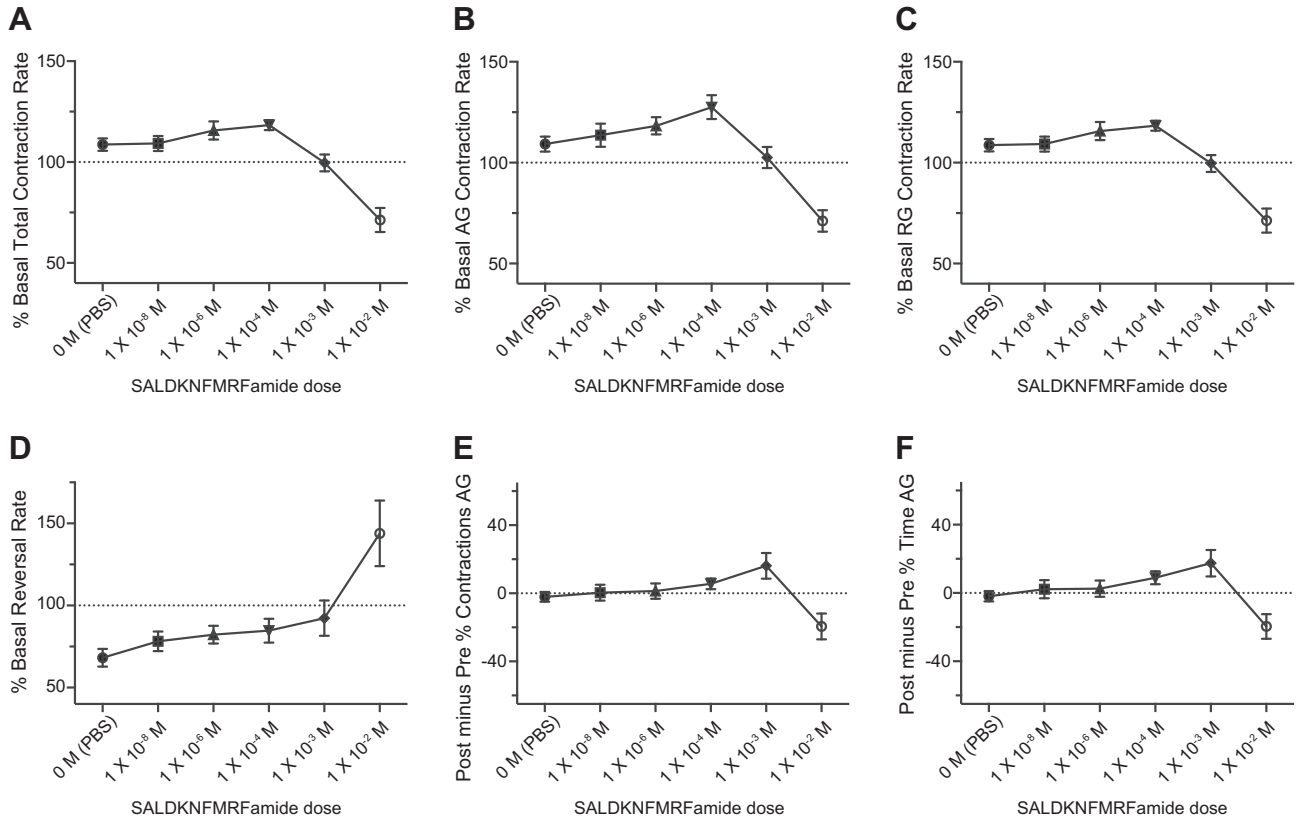

Fig. 7. Dose dependent effect of SALDKNFMRFamide injection on mosquito heart physiology at 10 min post-treatment. (A–C) Percent basal total (A), anterograde (B) and retrograde (C) contraction rates. (D) Percent basal frequency of heartbeat directional reversals. For each individual mosquito (A–D), the effect of treatment was calculated by dividing the post-treatment values by the pre-treatment values. (E) Difference in the percentage of contractions propagating in the anterograde direction after and before treatment. (F) Difference in the percentage of time spent contracting in the anterograde direction after and before treatment. For each individual mosquito (E, F), the effect of treatment was calculated by subtracting the value before treatment from the value after treatment (e.g., percent time anterograde after treatment minus percent time anterograde before treatment). For all graphs, the points mark the average and the whiskers denote the standard error of the mean. For a similar dose dependence graph that shows the effect of FMRFamide treatment at 10 min post-injection, see Fig. S8 in Supplementary file 1.

RF-containing peptides that are not produced by the *FMRFamide* gene (Taghert, 1999).

When considering the cardiomodulatory effects of FMRFamide and SALDKNFMRFamide, the strongest effects were seen at the higher dose, where both the heart rate and the directional proportionality of heart contractions were affected. FLPs were initially discovered in a bivalve because of the cardioacceleratory properties of FMRFamide (Price and Greenberg, 1977). Since, FLPs have been shown to have myotropic properties in a broad range of organisms (Nichols, 2003; Orchard et al., 2001; Walker et al., 2009). In insects, FMRFamide and other FLPs have been shown to be cardioacceleratory, be cardiodeceleratory, have no effect on heart physiology, or have an effect that is dependent on the presence of other molecules (Brown et al., 1986; Cuthbert and Evans, 1989; Duttlinger et al., 2003; Duve et al., 1993; Lee et al., 2012; Nichols, 2006; Nichols et al., 1999; Robb and Evans, 1990). Thus, the finding that FLPs possess myotropic properties in adult mosquitoes was expected. Moreover, it was interesting that both FMRFamide and SALDKNFMRFamide elicited similar effects on the mosquito heart. Specifically, both peptides elicited bimodal effects that were dependent on the dose: low doses were cardioacceleratory whereas high doses were cardioinhibitory. The finding that different doses of a specific FLP elicit dissimilar effects is not unprecedented. In *S. gregaria*, YGGFMRFamide is cardioacceleratory at lower doses but is inhibitory at higher doses (Cuthbert and Evans, 1989). However, unlike what was seen in our study, in *S. gregaria* the pattern induced by YGGFMRFamide is different from the pattern induced by FMRFamide and other FLPs.

Although the bimodal effects of FMRFamide and SALDKNFMRFamide in mosquitoes are clear, this pattern is only significant if it occurs at physiologically relevant concentrations. The endogenous concentration of FLPs in mosquitoes remains unknown, but based on studies in other invertebrates (Nagle, 1982; Robb and Evans, 1990), it is most likely that the cardioinhibitory activity of SALDKNFMRFamide occurs at a concentration that is higher than what is normally found in the mosquito hemocoel. Because the dose that yields a cardioacceleratory effect is more in line with (1) the doses that yield myotropic activity in other insects (Cuthbert and Evans, 1989; Duve et al., 1993; Maynard et al., 2013; Nichols, 2006; Nichols et al., 1999), (2) the measured concentration of FMRFamide containing peptides in other invertebrates (Nagle, 1982; Robb and Evans, 1990), and (3) the dose at which CCAP is cardioacceleratory in mosquitoes (Chen and Hillyer, 2013; Estevez-Lao et al., 2013), we hypothesize that, as pertains to cardiac physiology, SALDKNFMRFamide functions as a cardioacceleratory molecule.

Various neuropeptides are known to modulate heart contraction dynamics in insects (Chapman et al., 2013; Klowden, 2013). Some of these neuropeptides are highly conserved across taxa (e.g., CCAP) or contain conserved peptide sequences (e.g., FLPs) (Estevez-Lao et al., 2013; Lee et al., 2011; Nichols, 2003; Orchard et al., 2001; Walker et al., 2009). Here we describe the *FMRFamide* gene in *A. gambiae*. We show that the heart of the adult mosquito is modulated by at least one of the peptides encoded by this gene (SALDKNFMRFamide), and that the effect of this peptide is similar to the effect induced by the tetrapeptide (FMRFamide) that led to the discovery of this group myotropic molecules.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ygcen.2014.03.048.

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