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SIFamide peptides modulate cardiac activity differently in two species of *Cancer* crab

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ABSTRACT

The SIFamides are a broadly conserved arthropod peptide family characterized by the C-terminal motif –SIFamide. In decapod crustaceans, two isoforms of SIFamide are known, GYRKPPFNGSIFamide (Gly^1-SIFamide), which is nearly ubiquitously conserved in the order, and VYRKPPFNGSIFamide (Val^1-SIFamide), known only from members of the astacidean genus *Homarus*. While much work has focused on the identification of SIFamide isoforms in decapods, there are few direct demonstrations of physiological function for members of the peptide family in this taxon. Here, we assessed the effects of Gly^1- and Val^1-SIFamide on the cardiac neuromuscular system of two closely related species of *Cancer* crab, *Cancer borealis* and *Cancer irroratus*. In each species, both peptides were cardioactive, with identical, dose-dependent effects elicited by both isoforms in a given species. Threshold concentrations for bioactivity are in the range typically associated with hormonal delivery, *i.e.*, 10^{-9} to 10^{-8} M. Interestingly, and quite surprisingly, while the predicted effects of SIFamide on cardiac output are similar in both *C. borealis* and *C. irroratus*, frequency effects predominate in *C. borealis*, while amplitude effects predominate in *C. irroratus*. These findings suggest that, while SIFamide is likely to increase cardiac output in both crabs, the mechanism through which this is achieved is different in the two species. Immunohistochemical/mass spectrometric data suggest that SIFamide is delivered to the heart hormonally rather than locally, with the source of hormonal release being midgut epithelial endocrine cells in both *Cancer* species. If so, midgut-derived SIFamide may function as a regulator of cardiac output during the process of digestion.

1. Introduction

Central pattern generators (CPGs) are neural circuits that drive the muscle movements responsible for generating rhythmic motor behaviours, *e.g.*, locomotion, respiration, and mastication. As all animals must adapt their movements to varying conditions, both internal and environmental, CPGs must be functionally flexible on both physiological and evolutionary time scales (Katz, 2011, 2016). One mechanism for generating functional flexibility in CPG-effector systems is the alteration of neuronal and muscle properties by locally released and circulating chemical compounds (*e.g.*, Harris-Warrick, 2011; Marder et al., 2014, 2015; Daur et al., 2016; Dickinson et al., 2016; Cropper et al., 2017; Diaz-Rios et al., 2017; Schulz and Lane, 2017). This modulation allows for the conversion of a single "hard-wired" system into multiple functionally distinct systems; the specific functional CPG-effector system that results from modulation is dependent on the modulator or combination of modulators that are influencing it at a given moment in time. Modulatory systems (*i.e.*, chemical compounds and their cognate receptors) also provide a substrate for evolutionary flexibility in the functioning of CPG-effector systems.

Many classes of compounds have been shown to act as neuro/myomodulators, with peptides being the largest and most diverse of these groups (*e.g.*, Kastin, 2013). While some peptide families consist of a single isoform within a given taxon, others are comprised of multiple isoforms, which can be derived either from a common precursor protein or from the protein products of distinct paralog genes (reviewed in Christie et al., 2010). For some families for which multiple isoforms exist, the physiological actions of all members appear to be identical, or nearly so, on a given target in a given species (*e.g.*, Stemmler et al., 2007b; Ma et al., 2009; Szabo et al., 2011; Dickinson et al., 2015b), while for other peptide groups, isoform-specific actions have been noted (*e.g.*, Dickinson et al., 2015a,c, 2018).

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One CPG-effector system that is extensively modulated by both locally released and circulating peptides is the cardiac neuromuscular system of decapod crustaceans (reviewed in Cooke, 2002). This CPG-effector system consists of the cardiac ganglion (CG) and the musculature of the heart; unlike the myogenic vertebrate heart, the crustacean heart is neurogenic (Cooke, 2002). The decapod CG is one of the most numerically simple CPGs known, consisting of just nine neurons; these include five large motor neurons, whose activity directly drives the contractions of the heart musculature, and four small pre-motor neurons, whose activity is responsible for generating the regular bursting activity of the network (*e.g.*, Cooke, 2002). Peptides and other modulators, both locally released and hormonally delivered, can alter the output of the cardiac neuromuscular system by acting on the motor and/or pre-motor neurons of the CG, on the heart muscles, on the neuromuscular junction, or on any combination of these sites (*e.g.*, Miller and Sullivan, 1981; Sullivan and Miller, 1984; Wilkens et al., 2005; Fort et al., 2007a,b; Stevens et al., 2009; Dickinson et al., 2015a).

Members of approximately 35 different families of peptides have been identified in decapod species, including one group, the SIFamides, whose members are characterized by the conserved carboxyl-terminal motif –SIFamide (reviewed in Christie et al., 2010). The peptide GYRKPPFNGSIFamide (Gly^1-SIFamide) appears to be nearly ubiquitously conserved in members of the Decapoda (*e.g.*, Stemmler et al., 2007a), the sole known exception being members of the astacidean genus *Homarus*, where VYRKPPFNGSIFamide (Val^1-SIFamide) is the native isoform (Christie et al., 2006; Stemmler et al., 2007a). Members of the SIFamide family have been shown to be powerful modulators of the CPGs present in the stomatogastric nervous system (STNS), which controls the rhythmic movement of food through the foregut of decapods (Christie et al., 2006; Blitz et al., 2019); whether or not they are also cardioactive in this crustacean group, or members of the Arthropoda in general, remains unknown.

The study presented here focused on assessing the effects of SIFamide on the cardiac neuromuscular system of two closely related decapod species, namely the crabs *Cancer borealis* and *Cancer irroratus*, both native to the coastal waters off New England and Atlantic Canada. Three questions were investigated. First, are SIFamides cardioactive in *Cancer* species? Second, if so, do the effects elicited by one isoform differ from those of the other in a given species? Finally, if the SIFamides are bioactive, are the effects they elicit in the two species the same or different? As the data that follow will show, SIFamide was found to be cardioactive in *Cancer* crabs, with both Gly^1- and Val^1-SIFamide producing essentially identical effects on the heart in a given species. Interestingly, while the SIFamides elicit similar predicted effects on cardiac output in both *C. borealis* and *C. irroratus*, effects on heartbeat frequency predominate in the former, while effects on contraction amplitude predominate in the latter. These data suggest that, while SIFamide is likely to increase cardiac output in both crabs, the mechanism by which this is achieved is different in the two species. Mass spectrometry suggests that one source of SIFamide available to modulate the *Cancer* cardiac system is midgut epithelial endocrine cells. Immunohistochemistry suggests that SIFamide is not released within the CG or from either the X-organ-sinus gland (XO-SG) complex or the pericardial organ (PO), the two major neuroendocrine centers in *Cancer* species (reviewed in Christie, 2011), a finding that suggests that midgut-derived SIFamide may be responsible for regulating cardiac activity during the process of digestion.

2. Materials and methods

2.1. Animals

Jonah crabs, *C. borealis*, and Atlantic rock crabs, *C. irroratus*, were purchased from seafood retailers in Stonington, Brunswick, or Harpswell, ME, USA, and were maintained in either flow-through natural seawater tanks at ambient ocean temperature (10–14 °C) or recirculating natural seawater aquaria at 10–12 °C. Crabs were fed approximately weekly on a diet of crushed mussels or chopped squid and shrimp. In all cases, crabs were cold-anesthetized by packing in ice for 30–60 min before the removal of tissues for physiological, immunohistochemical, or mass spectrometric experiments.

2.2. Physiological recordings of heart contractions

After anaesthetization, the posterior dorsal region of the carapace, to which the heart is attached, was removed from the crab. The heart remained attached to the dorsal carapace to ensure that the extent to which it was stretched was similar to that in the intact crab. The dissected tissue, including the heart, was placed ventral side up in a deep dish lined with Sylgard 170 (Dow Corning, Midland, MI, USA) and filled with cold (~4 °C) physiological saline (composition in mM: 440 NaCl, 11 KCl, 13 $CaCl_2$, 26 $MgCl_2$, 10 HEPES; adjusted to pH 7.4–7.5 with NaOH) and pinned through the carapace. The posterior artery was cannulated with a piece of polyethylene tubing, which was inserted until it penetrated beyond the arterial valve. Saline was perfused through the heart at a rate of 2.5 mL/min (Dynamax Peristaltic Pump Model RP-1). A second perfusion line was directed across the top of the heart to help maintain temperature. Temperature in both perfusion lines was maintained between 10 and 12 °C with a Peltier temperature control system (CL100 bipolar temperature controller and SC-20 solution heater/cooler; Warner Instruments, Hamden, CT, USA).

To record heart contractions, a length of 6–0 suture silk was tied around the five anterior arteries and attached to a Grass FT03 force–displacement transducer at an angle of ~30 to 45°. Force was initially adjusted to 1.0 g; preparations were then allowed to stabilize for 1–2 h before the first application of a peptide. The output of the force transducer was amplified with an ETH-250 Bridge/Bio Amplifier (iWorx Amplifiers, Dover, NH, USA) and a Brownlee Precision Instrumentation amplifier (Model 440; San Jose, CA, USA). Data were recorded using a CED Power 1401 digitizer and Spike2 V6 (Cambridge Electronic Design, Cambridge, UK).

Peptides (Gly^1-SIFamide and Val^1-SIFamide) were custom synthesized by GenScript Corporation (Piscataway, NJ, USA). Peptides were dissolved in deionized water at a concentration of 10^{-3}M, and were kept frozen at −20 °C until needed. Just before use, peptides were diluted to the appropriate concentration in physiological saline. Each concentration (ranging from 10^{-9} to 10^{-6} M) of a peptide was perfused through the heart for 10 min; all peptide perfusions were followed by a 50-min wash in control saline to allow heart activity to return to baseline levels before the next peptide perfusion.

2.3. Data analysis

Heart contraction parameters were measured using the built-in functions of Spike2 and custom-written scripts in Spike2. Parameters measured included contraction frequency (calculated as 1/period), contraction amplitude, contraction duration (measured at half-maximal contraction amplitude), rise time, and relaxation time, as diagrammed in Fig. 1. Baseline values were calculated by averaging the values for the 200 s just before the peptide was applied; peptide averages were obtained by measuring values during a 200 s time period at the peak of the peptide effect, 200–300 s into the application. Additionally, we used the built-in functions of Spike2 to calculate the area under the curve for the same 200 s time frames; this value, transformed to area/unit time, reflects changes in contraction amplitude, frequency, and duration, and thus serves as an indirect indication of cardiac output. All data are presented as mean ± standard error of the mean (s.e.m.) in both the text and figures. To enable comparisons of preparations with different starting heart contraction parameters, values were normalized by calculating percent change for each parameter. These data were further analyzed and graphed using Microsoft Excel and Prism7 software (GraphPad Software, Inc., San Diego, CA, USA) and SPSS 25 (IBM

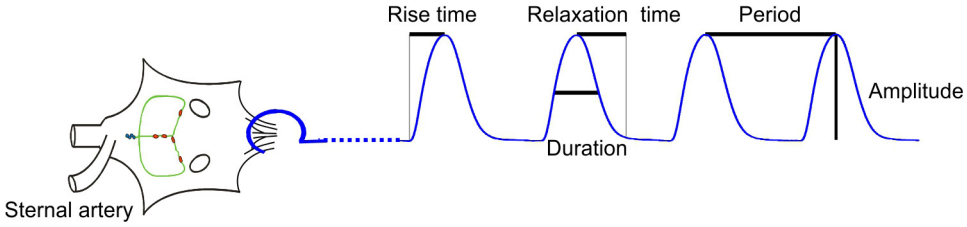

Fig. 1. Diagrammatic sketch of the crab heart and recording of force, indicating the parameters that were measured in each recording. The cardiac ganglion (green), consisting of 9 neurons, lies inside the heart. The sternal artery was cannulated, and peptides were introduced through the cannula. The five anterior arteries were tied to a force transducer at an angle of 30–45°. Parameters measured included cycle period (used to calculate heartbeat frequency), contraction amplitude (force), contraction duration (measured at half-maximal contraction amplitude), rise time, and relaxation time, as indicated on the diagram.

Corp., Armonk, NY, USA).

To determine whether baseline contraction frequency differed between the two species, we used an unpaired, two-tailed *t*-test (Prism 7). To determine whether each peptide application elicited a significant effect on heart rate parameters, we used single sample, two-tailed t-tests (Prism 7), comparing percent change in the peptide to a hypothetical value of 0. Threshold was considered to be just below the lowest concentration at which significant effects were recorded. To compare the responses of the two peptide isoforms (Gly^1-SIFamide and Val^1-SIFamide) in the two species (*C. borealis* and *C. irroratus*) across a range of peptide concentrations (10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M), we used 3-way ANOVAs implemented in SPSS. Sample sizes for these comparisons are shown in Table 1, with n representing the number of individual hearts tested from a single species at a given peptide concentration. Each heart was used for multiple concentrations of a single peptide; most hearts were perfused with multiple concentrations of each of the two peptides. To compare area/unit time, a factor that was measured only for Gly^1-SIFamide applications, across species and concentrations, we used a 2-way ANOVA in Prism 7.

2.4. Immunohistochemistry

To isolate the CG for immunohistochemistry, the posterior dorsal region of the carapace was removed from an anesthetized crab and the underlying heart was isolated and pinned ventral side up in a Petri dish lined with Sylgard 184 silicone elastomer (Dow Corning) containing chilled physiological saline. The pinned heart was opened along its ventral surface to expose the heart lumen; the CG was subsequently dissected from the surrounding heart musculature via manual microdissection. To obtain the PO, of which there are two per crab, the lateral walls of the pericardial chamber surrounding the heart were isolated and pinned in a Sylgard-lined deep glass dish filled with chilled physiological saline. The PO was then isolated from the musculature of the pericardial chamber wall via manual microdissection. To obtain the SG, of which there are two per crab, the eyestalks were isolated; the carapace was then split on two sides, opened and pinned in a Sylgard-lined Petri dish containing chilled physiological saline. The eyestalk ganglia, which contain the XO-SG system, were then isolated from surrounding musculature within the eyestalk.

Regardless of tissue type, immunohistochemistry was done as wholemounts. Specifically, tissues were pinned in Sylgard-lined Petri dishes and fixed for 12–24 h at 4 °C in a 4% paraformaldehyde solution (Electron Microscopy Sciences; Hatfield, PA, USA; catalog #15710) in 0.1 M sodium phosphate buffer (*P*). After fixation, tissues were rinsed five times at 1-h intervals at room temperature (18–20 °C) in *P* containing 0.3% Triton-X 100 (Sigma-Aldrich; catalog # X100-100ML) (*P-Triton*). Rinsed tissues were incubated for approximately 72 h at 4 °C in a rabbit polyclonal anti-Val^1-SIFamide antiserum diluted 1:500 in *P-Triton* containing 10% normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, West Grove, PA, USA; catalog #017-000-121); the development of the anti-SIFamide antibody is described in detail in Christie et al. (2006). Following primary antibody incubation, tissues were again rinsed five times at 1-h intervals at room temperature in *P-Triton*; they were then incubated for 12–24 h at 4 °C in Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen Corporation, Carlsbad, CA, USA; catalog #A-21206) diluted 1:300 in *P-Triton* containing 10% NDS. After secondary antibody incubation, tissues were rinsed five times at 1-h intervals at room temperature in *P*, then mounted between a glass microscope slide and coverslip in Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA; catalog #H-1000) for imaging. Incubation in secondary antibody and all subsequent processing was conducted in the dark; slides were stored in the dark at 4 °C until examined for immunoreactivity. Although the antibody used here was generated against Val^1-SIFamide, it cross-reacts with Gly^1-SIFamide (Christie et al., 2006), the native *Cancer* isoform (Stemmler et al., 2007a). Moreover, preadsorption of the Val^1-SIFamide antibody with the Gly^1 isoform of the peptide abolishes all immunolabeling in *Cancer* tissues that have been shown to contain the peptide (Christie et al., 2007; Blitz et al., 2019).

Mounted immunolabeled tissues were examined using either a Zeiss Axiovert 200 epifluorescence microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) or an Olympus Fluoview 1000 confocal system (Olympus America, Center Valley, PA, USA). The Axiovert 200 was equipped with EC Plan-NEOFLUAR 10×/0.3, LD Plan-NEOFLUAR 20×/0.4, and LD Plan-NEOFLUAR 40×/0.6 objective dry lenses, an EXFO X-Cite Series 120 halide arc lamp (EXFO Photonic Solutions Inc., Mississauga, Ontario, Canada), and a standard Zeiss FITC filter set. The Olympus Fluoview 1000 confocal system consisted of an Olympus IX-81 inverted microscope, 10x Uplan S Apo, 20× Uplan FLN, and 40× Uplan S Apo dry objective lenses, HeNe and multi-Ar lasers, a manufacturer-supplied Alexa Fluor 488 filter set, and manufacturer-supplied image-processing software.

Table 1
Sample sizes (n) for 3-way ANOVA.

	10^{-9} M	10^{-8} M	10^{-7} M	10^{-6} M
C. borealis; Gly^1-SIFamide	9	10	10	10
C. borealis; Val^1-SIFamide	9	12	11	11
C. irroratus; Gly^1-SIFamide	13	14	14	14
C. irroratus; Val^1-SIFamide	9	9	11	11

2.5. Mass spectrometry

2.5.1. Preparation of midgut tissues for direct tissue mass spectrometry

For direct tissue matrix-assisted laser desorption/ionization-Fourier transform mass spectrometry (MALDI-FTMS), midguts were removed from the thorax of cold-anesthetized crabs and pinned in a Sylgard-lined Petri dish containing physiological saline; small pieces of anterior midgut caecum (AMC) and posterior midgut caecum (PMC) were isolated via manual microdissection. Tissue fragments were rinsed sequentially in two 12 µL droplets of 0.75 M fructose (Sigma-Aldrich, St. Louis, MO, USA; 99%), placed on one face of a ten-faceted probe tip, and then sliced 10–20 times with a 0.1 mm needle. The macerated tissue was then gathered together and covered with a 0.5 µL droplet of 1.0 M 2,5-dihydroxybenzoic acid (DHB; Sigma-Aldrich; 98%, sublimed prior to use), prepared in 1:1 acetonitrile (Fisher Scientific, Pittsburg, PA, USA; HPLC grade) and water containing 2% (v/v) phosphoric acid.

2.5.2. Preparation of midgut tissue extracts

Midgut tissue extracts were prepared by placing a piece of AMC or PMC in a 0.6 mL low retention microcentrifuge tube with 50 µL of extraction solvent (a %[v/v] mixture of 30% deionized water, 65% methanol [CH_3OH; HPLC-grade; Fisherbrand], and 5% glacial acetic acid [CH_3CO_2H; reagent grade; Sigma-Aldrich, ≥99%]) after sequential rinsing in two, 12 µL droplets of 0.75 M D-fructose solution. The tissues were homogenized by repeated slicing with spring scissors, followed by sonication for 5 min and centrifugation at 15 K rpm for 5–15 min. The supernatant was removed from the sample and placed in another 0.6 mL tube. Samples were delipidated prior to analysis by adding 20 µL of nanopure water and 25 µL chloroform (NMR-grade $^{13}CDCl_3$; Cambridge Isotope Laboratories, Tewksbury, MA, USA) to the samples. The two layers were sonicated for 2 min and centrifuged for 10 min. The bottom organic layer was removed and discarded. Chloroform (40 µL) was added and the extraction was repeated. The aqueous layer was concentrated to dryness in a SpeedVac vacuum concentrator (UVS400 Universal Vacuum System; Thermo Electron Corporation, Waltham, MA, USA) at 36 °C. Once dry, the extract was reconstituted to a total volume of 50 µL in 1:1 ACN: H_2O and analyzed by MALDI-FTMS. For MALDI-FTMS analysis, 0.5 µL of the extract was mixed with 0.5 µL of DHB matrix on one face of the MALDI probe and the extract-matrix mixture was allowed to co-crystallize.

2.5.3. MALDI-FTMS analysis

All midgut samples were analyzed using a HiResMALDI Fourier transform mass spectrometer (IonSpec, Lake Forest, California, USA) equipped with a Cryomagnetics (Oak Ridge, TN, USA) 4.7 Tesla actively shielded superconducting magnet (Department of Chemistry, Bowdoin College, Brunswick, ME, USA) as described for neural tissues in Christie et al. (2006). Internal mass calibration was carried out using selective in-cell accumulation of calibrant as previously described in Stemmler et al. (2005). Poly(propylene glycol) 725 and 2000 (PPG; Sigma-Aldrich) was used as the calibrant for most measurements. For sustained off-resonance irradiation collision-induced dissociation (SORI-CID) experiments, argon was used as the collision gas, the frequency offset was set to −1.8% of the reduced cyclotron frequency and the voltage amplitude was in the range of 6–8.5 V_{bp}. SORI-CID spectra were calibrated externally, with a one-point adjustment based upon a $[MH\text{-}NH_3]^+$ fragment mass.

2.5.4. Figure production

For the production of MALDI-FTMS figures, mass spectral traces were calibrated and exported from the Boston University Data Analysis (BUDA) program and then labeled in CorelDRAW (Corel Corporation, Ottawa, Ontario, Canada).

3. Results

3.1. Unmodulated heart rates differ between Cancer borealis and Cancer irroratus

In the absence of exogenously applied modulators, contraction frequencies in isolated *C. borealis* and *C. irroratus* hearts varied among individuals, with more than a two-fold range of heartbeat frequencies (Fig. 2). Mean contraction frequency in *C. borealis* (0.31 ± 0.03 Hz) was lower than that of *C. irroratus* (0.61 ± 0.04 Hz) (unpaired *t*-test, $p < 0.0001$, $n = 13$ for each species).

3.2. Gly[1]- and Val[1]-SIFamide exert similar cardiotropic effects in Cancer borealis and Cancer irroratus

Perfusion of SIFamides through the isolated whole heart of the two *Cancer* species examined here resulted in an enhancement of cardiac functioning. Both contraction amplitude and contraction frequency increased during peptide perfusion (Fig. 3). To determine the threshold

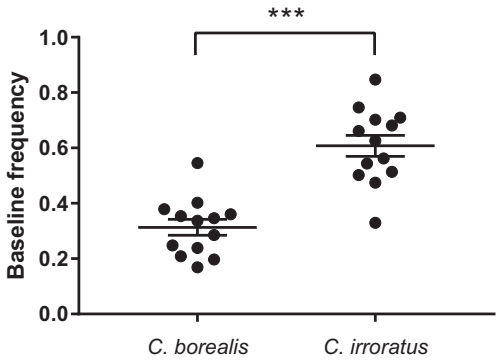

Fig. 2. Baseline heartbeat frequency was lower in *C. borealis* than in *C. irroratus*. Although heartbeat frequency measured in control saline varied over a greater than 2-fold range in both species, it was lower (unpaired *t*-test, $p < 0.0001$, $n = 13$ for each species) in *C. borealis* (mean 0.31 Hz) than in *C. irroratus* (mean 0.61 Hz). Coefficients of variation were similar in the two species (32.9% in *C. borealis*; 22.6% in *C. irroratus*). Error bars indicate standard error (s.e.m.).

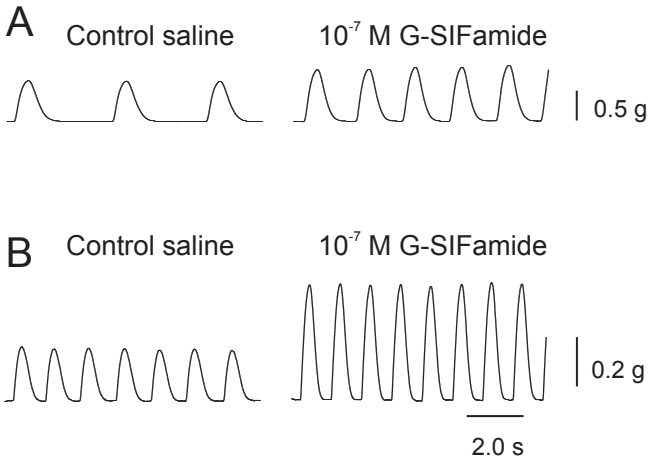

Fig. 3. Gly[1]-SIFamide increased contraction amplitude and frequency in both *Cancer borealis* and *Cancer irroratus*. Recordings of contractions in control saline and in 10^{-7} M Gly[1]-SIFamide illustrate the substantial increase in contraction frequency in *C. borealis* (A) and in contraction amplitude in *C. irroratus* (B). Smaller increases in contraction amplitude in *C. borealis* and in contraction frequency in *C. irroratus* can also be seen. Heartbeat frequency in control saline was higher in *C. irroratus* than in *C. borealis*.

at which the SIFamides elicited effects on the heart, we perfused both Gly[1]-SIFamide and Val[1]-SIFamide through the hearts of *C. borealis* and *C. irroratus* at concentrations of 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M (Fig. 4). In *C. borealis*, threshold for Gly[1]-SIFamide appeared to be at or below 10^{-9} M, since 10^{-9} M Gly[1]-SIFamide elicited significant effects (*i.e.*, percent change from baseline was significantly different from 0) on both contraction frequency (single sample *t*-test, $p = 0.008$, $n = 9$; Fig. 4A1) and contraction duration (single sample *t*-test, $p = 0.017$, $n = 9$; Fig. 4C1). Contraction amplitude was somewhat less sensitive to Gly[1]-SIFamide, with significant effects of the peptide recorded at concentrations of 10^{-8} M and above (Fig. 4B1). The alternative SIFamide isoform, Val[1]-SIFamide, elicited significant effects on each of the three major contraction parameters in *C. borealis* at concentrations of 10^{-8} M and above, suggesting a threshold for that peptide between 10^{-8} and 10^{-9} M (Fig. 4A1, B1, C1). In *C. irroratus*, thresholds for the cardiotropic effects of both peptides on all three major contraction parameters, *i.e.*, frequency, amplitude, and duration, were between 10^{-8} and 10^{-9} M, with 10^{-8} M, but not 10^{-9} M, SIFamide exerting significant effects in all cases (Fig. 4A2, B2, C2).

Although the lowest concentration at which Gly[1]-SIFamide elicited

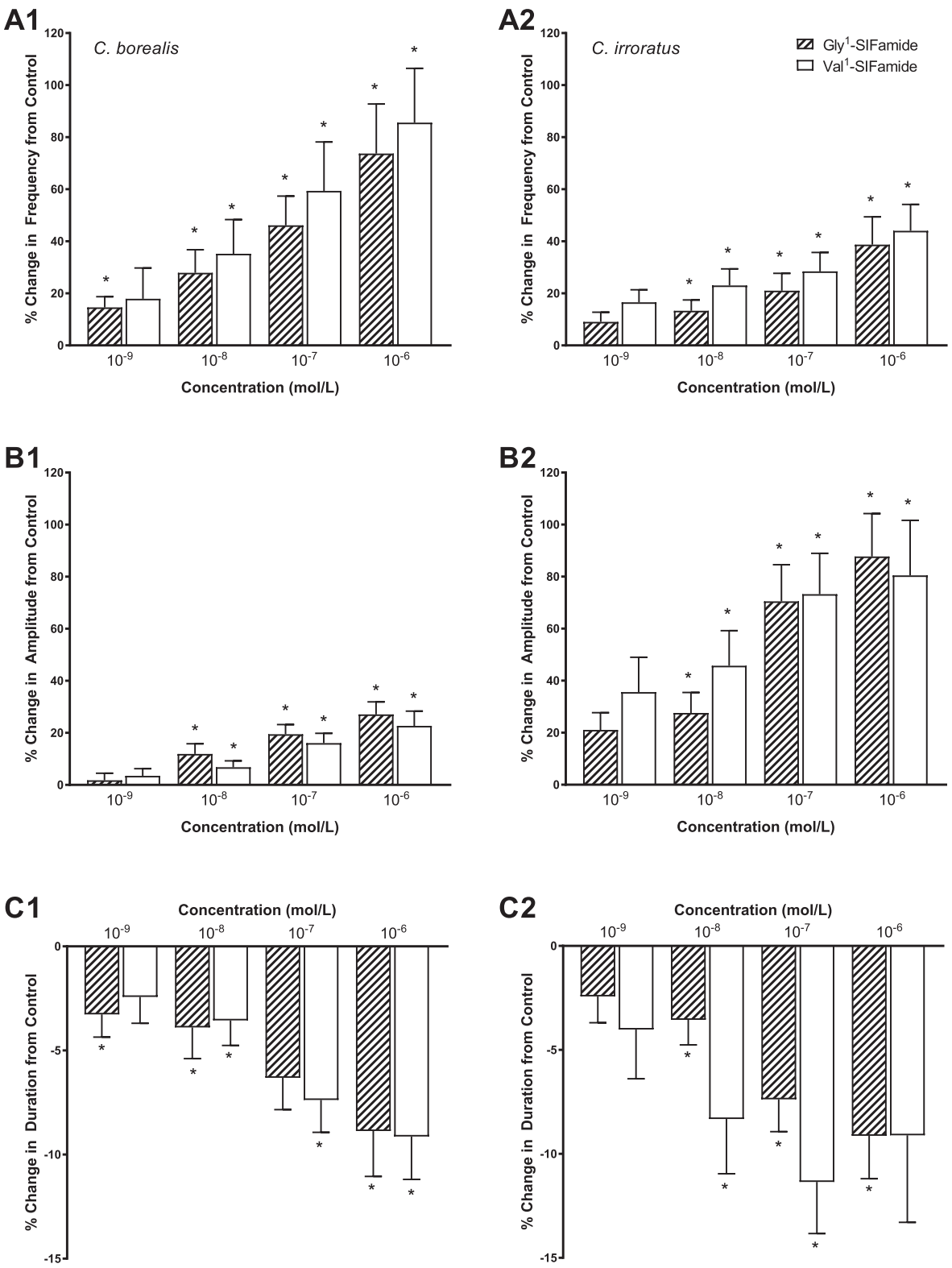

Fig. 4. Both Gly[1]-SIFamide and Val[1]-SIFamide elicited dose-dependent effects on the frequency, amplitude, and duration of heart contractions in *C. borealis* and in *C. irroratus*. For all parameters in both species, there were no significant differences between the effects of Gly[1]-SIFamide and Val[1]-SIFamide (3-way ANOVA, $p > 0.05$). Thresholds for effects of both peptides were less than 10^{-8} M, with both peptides eliciting significant effects on each species at a concentration of 10^{-8} M or less. In *C. borealis*, threshold appeared to be somewhat lower for Gly[1]-SIFamide. Gly[1]-SIFamide, but not Val[1]-SIFamide, elicited significant effects at concentrations of 10^{-9} M for both frequency (A1) and duration (C1). (A1, A2) The SIFamides elicited dose-dependent increases in contraction frequency in both species; the increase was greater in *C. borealis* than in *C. irroratus* (3-way ANOVA: concentrations, $p < 0.001$; species, $p < 0.001$). (B1, B2) The SIFamides elicited dose-dependent increases in contraction amplitude in both species; the increase was greater in *C. irroratus* than in *C. borealis* (3-way ANOVA: concentrations, $p < 0.001$; species, $p < 0.001$). (C1, C2) The SIFamides elicited dose-dependent decreases in contraction duration in both species; this decrease did not differ significantly between the two species (3-way ANOVA: concentrations, $p < 0.001$; species, $p > 0.05$). * indicates values significantly different from 0, single sample *t*-test. Sample sizes: ≥9; see Table 1 for details. Error bars indicate standard error (s.e.m.).

an effect in *C. borealis* was one order of magnitude below that at which Val^1-SIFamide elicited an effect, the effects of the two peptides on the *C. borealis* heart did not otherwise differ (3-way ANOVA, $p > 0.05$ for all parameters; Fig. 4A1, B1, C1). Similarly, there were no differences between the effects of the two SIFamide isoforms in *C. irroratus* (3-way ANOVA, $p > 0.05$ for any of the three parameters; Fig. 4A2, B2, C2). For both peptides and both species, the effects of the SIFamides on contraction frequency, amplitude and duration were highly dose-dependent (3-way ANOVA, $p < 0.001$ for all parameters; Fig. 4).

3.3. SIFamide appears to exploit different mechanisms in Cancer borealis and Cancer irroratus

Although SIFamides were clearly cardiotropic in both *Cancer* species tested, the mechanisms by which the peptides modulated ongoing cardiac activity appeared to differ. Specifically, SIFamides elicited large increases (up to > 80%) in contraction frequency, but only small increases (up to ~20%) in contraction amplitude in *C. borealis*. The opposite was the case in *C. irroratus*, in which we recorded relatively small (up to ~40%) increases in contraction frequency, but large (up to ~80%) increases in contraction amplitude. The differences between species in response to the peptides for both contraction amplitude and frequency were highly significant (3-way ANOVA, $p < 0.001$ for both parameters; Fig. 4A1, A2, B1, B2).

Associated with the increases in frequency and amplitude elicited by the SIFamides were decreases in contraction duration. These decreases, while significantly different from 0 at concentrations of 10^{-8} M and higher in both species, were relatively small, reaching a maximum of ~10%. Moreover, although the decreases were dose-dependent, they did not differ between the two species (3-way ANOVA, $p > 0.05$; Fig. 4C1 and C2).

The duration of the contraction is determined by both the time to peak ('rise time') and the time required for the heart to relax back to baseline force ('relaxation time'). Interestingly, the two *Cancer* species appeared to exploit these parameters differently to achieve a decrease in contraction duration. Although the only decrease in rise time that was significantly different from 0 was the response to 10^{-6} M Gly^1-SIFamide (Fig. 5A), the change in rise time elicited by the peptides differed between the two species (3-way ANOVA, $p = 0.042$; Fig. 5A1, A2), but not across concentrations of the peptides (3-way ANOVA, $p > 0.05$; Fig. 5A1 and A2). In contrast, all decreases in relaxation time in *C. irroratus*, and all except those to 10^{-9} M peptide in *C. borealis* were significantly different from 0 (single sample t-tests, $p < 0.05$; Fig. 5B1 and B2). Moreover, the changes in relaxation time also differed between the two species (3-way ANOVA, $p = 0.021$; Fig. 5B1 and B2). Thus, although the decreases in contraction duration were due largely to decreases in relaxation time in both species, this effect was more pronounced in *C. irroratus* than in *C. borealis*.

These data suggest that the SIFamides increase cardiac output in both species, but use different mechanisms to do so. Although we have not directly measured this parameter, we calculated the area under the curve per unit time to provide an indirect indication of cardiac output. Area per unit time incorporates contraction frequency, amplitude, and duration, and thus can be used as a proxy for cardiac output. Because the effects of Gly^1-SIFamide and Val^1-SIFamide were not different for either species, we chose to calculate this value only for Gly^1-SIFamide, the native crab isoform. Interestingly, Gly^1-SIFamide elicited increases in area per unit time in both species, at all concentrations tested (*i.e.*, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M; Fig. 6). These increases did not differ between the two species.

3.4. Sites of SIFamide release in Cancer crabs

The threshold concentrations for the physiological effects of SIFamide on the cardiac neuromuscular systems of both *C. borealis* and *C. irroratus* were, on average, approximately 10^{-9}–10^{-8} M, which is within the range that has been measured in the hemolymph for other peptides in arthropods (*e.g.*, Robb and Evans, 1990; Elia et al., 1993; Žitňan et al., 1999; Fastner et al., 2007; Kang et al., 2014). One site of hormonal release of SIFamide in *Cancer* crabs is the epithelial endocrine cells of the anterior midgut/AMC (Christie et al., 2007), which are located beneath and in close proximity to the heart. In all *Cancer* species thus far investigated, SIFamide-immunopositive cells have been found in these portions of the digestive tract (Christie et al., 2007). It is possible that SIFamide may also be released within the *Cancer* CG itself, either from the motor or pre-motor neurons, or from axons projecting to the ganglion from neurons located elsewhere in the crab nervous system. It is also possible that SIFamide may be released into the hemolymph from one or both of the two major neuroendocrine organs of the crab, *i.e.*, the XO-SG and PO (*e.g.*, Christie, 2011). To determine whether SIFamide might modulate the cardiac neuromuscular systems of *C. borealis* and *C. irroratus* via local release within the CG and/or via release into the hemolymph from the XO-SG and PO, immunohistochemistry using an antibody generated against Val^1-SIFamide was conducted on all three neural tissues. No consistent SIFamide immunolabeling was seen in any of these three sites in either *C. borealis* or *C. irroratus* (n = 5 preparations/tissue/species).

3.5. Mass spectrometric identification of Gly^1-SIFamide in the anterior midgut of Cancer irroratus

To determine if authentic Gly^1-SIFamide was present in *C. irroratus* midgut tissues, as it is in the midgut of *C. borealis* (Christie et al., 2007), we conducted direct tissue MALDI-FTMS on samples isolated from both the AMC and PMC ($n \geq 3$). In the spectra collected from small pieces of the AMC ($n \geq 3$), we consistently detected a peak at *m/z* 1381.74 (Fig. 7A), a mass consistent with that of Gly^1-SIFamide (mass measurement error = 1.1 ppm). The peak at *m/z* 1381.74 was also detected in the analysis of AMC tissue extracts (Fig. 7C). The identification of this peak as Gly^1-SIFamide was further supported by measurement of an MS/MS spectrum for the isolated and dissociated signal at *m/z* 1381.74. The MS/MS spectrum (Fig. 7E) showed b-type ions, which contain the N-terminus of the peptides, and y-type ions, which contain the C-terminus, that showed excellent agreement, in terms of types of detected ions and relative ion abundance, with those observed in MS/MS spectra of Gly^1-SIFamide standards (data not shown).

Analysis of the PMC ($n \geq 3$ samples), using both direct tissue (Fig. 7B) and tissue extracts (Fig. 7D) for sample preparation, showed an intense peak appearing at *m/z* 934.49, which was consistently detected at a high relative abundance. The *m/z* 934.49 peak was identified as *Cancer borealis* tachykinin-related peptide Ia (CabTRP Ia; Christie et al., 1997) based upon exact mass measurements. However, spectra of the PMC samples showed no indication of a peak corresponding to Gly^1-SIFamide at *m/z* 1381.74, suggesting that Gly^1-SIFamide is localized to the anterior midgut/AMC, as has been reported previously for other *Cancer* species, including *C. borealis* (Christie et al., 2007).

4. Discussion

4.1. SIFamide is a cardioactive peptide in Cancer crabs

Although members of the SIFamide family are broadly conserved in arthropods, relatively little is known about the physiological roles played by members of this peptide family (*e.g.,* Verleyen et al., 2004, 2009). In the fruit fly, *Drosophila melanogaster*, targeted cell ablation and RNA interference (RNAi) have shown that SIFamide plays a role in the regulation of adult courtship behavior, with removal of peptide resulting in male flies performing indiscriminant courtship directed at both sexes and females exhibiting hyperreceptivity (Terhzaz et al., 2007; Sellami and Veenstra, 2015). Similarly, cell ablation/RNAi experiments have shown that the SIFamide signaling system promotes

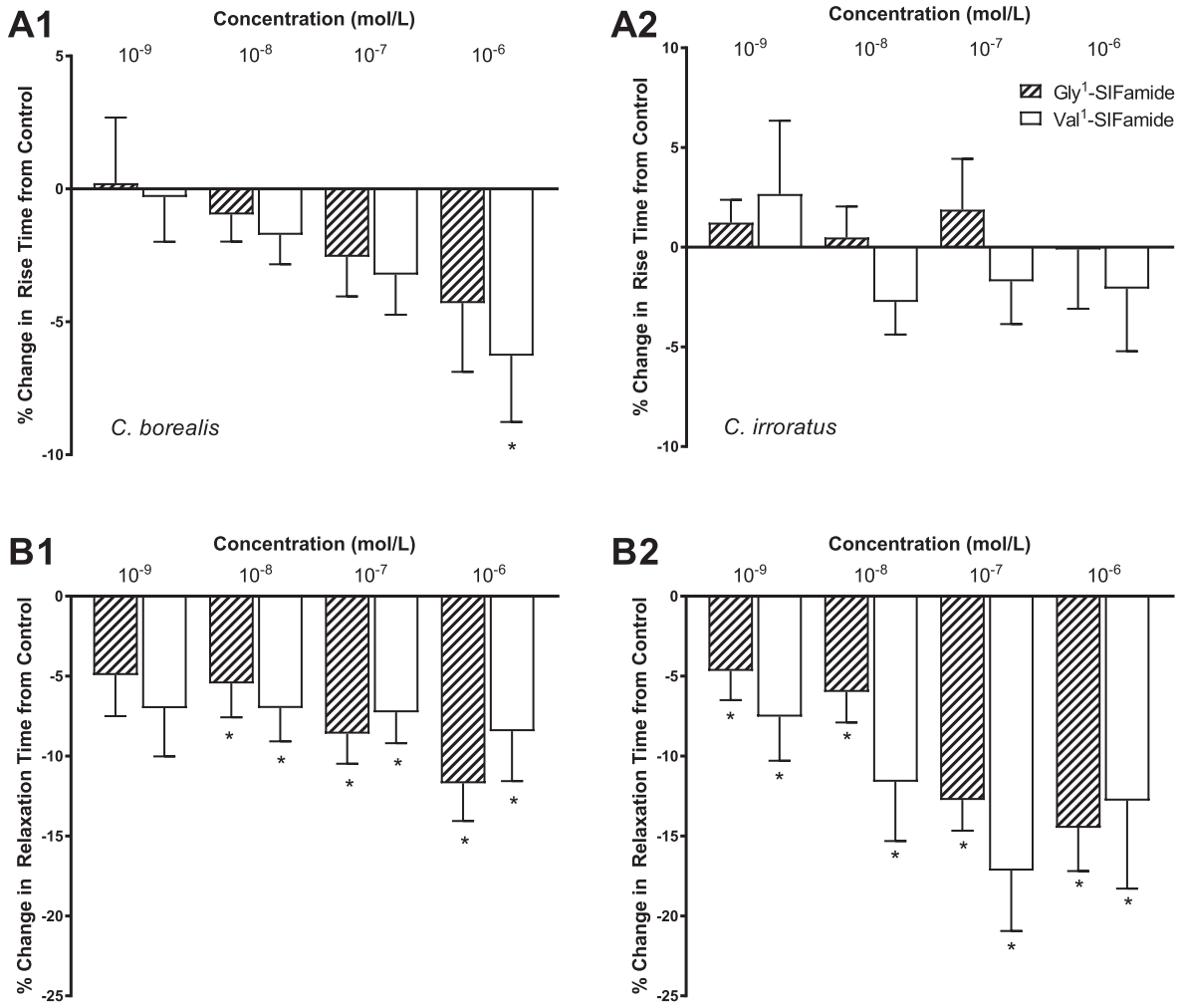

Fig. 5. The decrease in contraction duration elicited by the SIFamides was due largely to decreases in the duration of relaxation from peak contraction back to baseline. (A1, A2) Rise time decreased significantly only in *C. borealis* during perfusion of 10^{-6} M Val^1-SIFamide. There were no significant differences between the effects of the two peptides or across peptide concentrations; there were, however, differences between the two species (3-way ANOVA: peptides, $p > 0.05$; concentrations, $p > 0.05$; species, $p = 0.020$). (B1, B2) Relaxation time decreased significantly during SIFamide perfusion in nearly all concentrations of both peptides, although neither peptide elicited a significant change when perfused at a concentration of 10^{-9} M in *C. borealis*. Although these decreases in relaxation time (like those in duration) are relatively small, with all means being less than 15% change, the effects of the peptides on relaxation time were dose-dependent and differed between the two species (3-way ANOVA: peptides, $p > 0.05$; concentrations, $p = 0.004$; species, $p = 0.012$). Sample sizes: ≥9; see Table 1 for details. Error bars indicate standard error (s.e.m.).

sleep in *Drosophila* (Park et al., 2014); the SIFamide signaling system of *Drosophila* also appears to be involved in integrating orexigenic and anorexigenic signals to sensitize sensory circuits, promoting appetitive behavior and increasing food intake (Martelli et al., 2017). SIFamide has also been shown to activate hindgut motility in a dose-dependent fashion in the tick, *Ixodes scapularis* (Simo and Park, 2014). In crustaceans, SIFamide has been implicated in the modulation of aggression in the prawn, *Macrobrachium rosenbergii*, where injection of the peptide into the hemolymph enhanced aggressive behavior between interacting pairs of males (Vazquez-Acevedo et al., 2009). With respect to the modulation of crustacean CPG-effector systems, SIFamide has been shown to be a powerful modulator of the CPGs present in the STNS, which controls the rhythmic movement of food through the foregut. In the lobster, *H. americanus*, and the crab, *C. borealis*, application of SIFamide activated/enhanced both the pyloric and gastric mill rhythms, which control the rhythmic movements of the pyloric filter and teeth of the gastric mill, respectively, in similar, though non-identical manners (Christie et al., 2006; Blitz et al., 2019). In the study presented here, we show, for the first time, that members of the SIFamide family are also cardiotropic, with both Gly^1- and Val^1-SIFamide enhancing output of the hearts of both *C. borealis* and *C. irroratus*.

4.2. In Cancer species, the two known decapod SIFamides (Gly^1- and Val^1-SIFamide) elicit essentially identical effects on the heart

Many peptide families consist of multiple isoforms, either among species and/or within a given species. In some cases, it appears that all isoforms within a given family elicit the same physiological responses on a given target in a given species, whereas for others there appear to be isoform-specific differences in bioactivity. For example, in the STNS of both *C. borealis* and *H. americanus*, members of the pyrokinin family all activate/enhance the gastric mill motor pattern in an essentially identical manner, and none alter the activity of pyloric motor pattern (Saideman et al., 2006; Dickinson et al., 2015b). In contrast, only one of the pyrokinins tested on the *H. americanus* cardiac neuromuscular system was found to be bioactive (Dickinson et al., 2015c). Isoform-specific effects have also been noted on the *H. americanus* cardiac neuromuscular system for members of the FMRFamide-like peptide and allatostatin-C families (Dickinson et al., 2015a, 2018).

In decapod crustaceans, two isoforms of SIFamide are known: Gly^1-

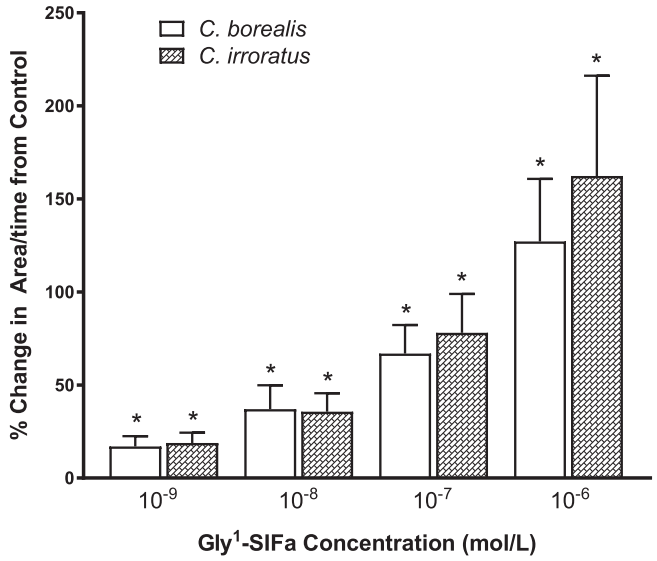

Fig. 6. Area/unit time, used here as an indirect indication of cardiac output, increased similarly in the two *Cancer* species in response to the native crab SIFamide, Gly^1-SIFamide. Increases in area/unit time, which is influenced by contraction amplitude, frequency, and duration, were dose-dependent (2-way ANOVA; $p < 0.0001$). This parameter increased at all concentrations tested, from 10^{-9} M to 10^{-6} M, but did not differ between the two species (2-way ANOVA, $p > 0.05$). * indicates values significantly different from 0, single sample *t*-test Sample size: n = 9. Error bars indicate standard error (s.e.m.).

SIFamide, which appears to be nearly ubiquitously conserved within the order (Stemmler et al., 2007a), and Val^1-SIFamide, which has thus far been found only in members of the genus *Homarus*, *i.e.*, *H. americanus* and *Homarus gammarus* (*e.g.*, Christie et al., 2006; Stemmler et al., 2007a). Here, we found that the two crustacean SIFamide isoforms are cardioactive in both *C. borealis* and *C. irroratus*. Five different parameters of heartbeat were measured in each species for each peptide: frequency, amplitude, duration, rise time, and relaxation time. No statistically significant differences between the two peptide isoforms were seen for any of the measured parameters in either crab species. This finding suggests the glycine vs. valine residue at position 1 has no effect on SIFamide bioactivity, at least on the cardiac neuromuscular systems of the two *Cancer* species studied here. Whether or not this finding will hold true for other targets of SIFamide in *C. borealis* and *C. irroratus*, *e.g.*, the stomatogastric neuromuscular system, remains to be determined. Similarly, it will be interesting to see if the same results hold for the cardiac neuromuscular systems of *H. americanus* and/or *H. gammarus*, the only species in which Val^1-SIFamide is the native SIFamide isoform.

4.3. Predicted increases in cardiac output in both Cancer borealis and Cancer irroratus appear to result from different mechanisms

Although we did not measure cardiac output *per se*, our data strongly suggest that SIFamide elicits an increase in cardiac output in both *C. borealis* and *C. irroratus*. While contraction duration decreased in both species, this decrease was proportionally much smaller than the increases seen in other parameters, specifically frequency and/or amplitude. Even with a small decrease in contraction duration, the increased contraction amplitude would be predicted to drive an increase in stroke volume. Coupled with an increase in contraction frequency, cardiac output is predicted to increase substantially. As an indirect measure of cardiac output, we measured the area under the force curve per unit time; this value increased in a dose-dependent manner in both species. Interestingly, although threshold for each of the individual contraction parameters was generally between 10^{-8} and 10^{-9} M, this value differed from zero even at 10^{-9} M in both species, suggesting that the threshold for effects on cardiac output is below 10^{-9} M. Moreover, the area per unit time did not differ between the two species, suggesting that the peptide exerts similar functional effects in both species.

Interestingly, the mechanisms utilized by the two species to effect this increase in cardiac output were strikingly different. In *C. borealis*, SIFamides elicited a large increase in contraction frequency and a much smaller increase in contraction amplitude, while the opposite was the case in *C. irroratus*, in which the peptides elicited a large increase in contraction amplitude and a smaller increase in contraction frequency. Interestingly, this pattern correlates with the baseline contraction frequencies recorded in the two species. Baseline frequency was significantly higher in *C. irroratus* than in *C. borealis*; in *C. borealis*, even in the preparations with the largest increases in contraction frequency, frequency never exceeded 0.7 Hz. In contrast, mean baseline contraction frequency in *C. irroratus* was over 0.6 Hz; in the presence of SIFamide, contraction frequencies reached values as high as 1.1 Hz. Together, these data suggest that the range for increases in frequency may be limited in *C. irroratus* relative to *C. borealis*.

Two aspects of cardiac function might contribute to the limited range for an increase in frequency in *C. irroratus*. First, cardiac contractions are driven by the output of the CG, which lies within the decapod heart (reviewed in Cooke, 2002). Four small pre-motor neurons and five large motor neurons comprise the pattern generator that controls cardiac contractions. These neurons, which are connected by both electrotonic and chemical synapses, generate repeated driver potentials. The driver potentials in the motor neurons in turn trigger action potentials that travel down the motor nerves to innervate the muscles, where they trigger graded contractions. Pacemaker potentials in the pre-motor neurons are responsible for depolarizations between driver potentials, and thus help to determine the frequency of the heart contractions (see Cooke, 2002) for review). One important aspect of driver potentials is that they, like action potentials, have refractory periods; in conjunction with this, their amplitude and threshold change with time after a previous driver potential. Consequently, there is a maximum rate at which driver potentials of an amplitude sufficient to elicit cardiac contractions can occur. Although there are no reports of intracellular recordings from CG neurons in either of the two species examined here, the rate at which the ganglion could generate an output sufficient to drive full cardiac contractions is one factor that might limit the extent to which increases in cardiac output could be driven by changes in contraction frequency.

Second, effects at the neuromuscular junction or the muscle itself might limit the range for increases in frequency. The neuromuscular transform is a non-linear function that describes the patterns of muscle contraction that will result from a particular set of neuronal inputs (Brezina et al., 2000; Brezina and Weiss, 2000; Williams et al., 2013). This function encompasses multiple physiological factors, including presynaptic transmitter release, post-synaptic calcium dynamics, and other processes that take place at the neuromuscular junction and within the muscle (Brezina et al., 2000; Brezina and Weiss, 2000; Williams et al., 2013). Although the neuromuscular transform has not been studied in *Cancer* crabs, it has been characterized in another decapod crustacean, the lobster, *H. americanus* (Williams et al., 2013). In this species, as frequency increases, especially to values approaching 1.0 Hz, contraction amplitude decreases from its maximum, and requires a longer duty cycle of neuronal firing to achieve that contraction amplitude. Because we do not have recordings of the neuronal output that drives the heart contraction in these crab species, we cannot calculate duty cycle directly. Nonetheless, if we assume similar neuromuscular transforms for these species, it is clear that an increase in frequency from a relatively high baseline would be relatively ineffective in increasing contraction amplitude; increases in burst duration, and hence in duty cycle of neuronal firing, would likely be more effective.

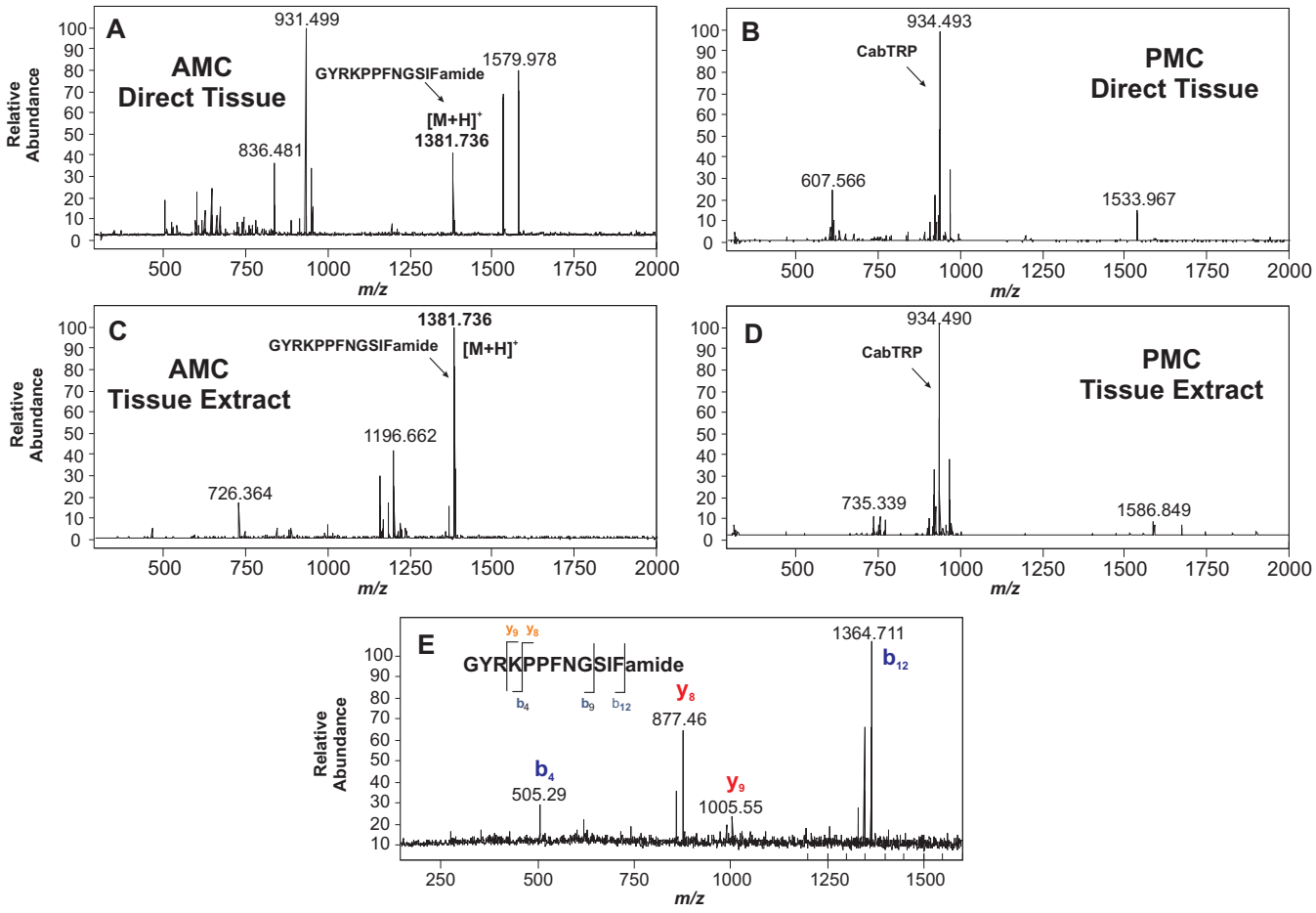

Fig. 7. Direct MALDI-FTMS analysis of *C. irroratus* midgut tissues suggests the presence of Gly^1-SIFamide in the anterior midgut caecum (AMC), but not in the posterior midgut caecum (PMC). *C. irroratus* midgut tissues were analyzed by MALDI-FTMS, using DHB as the matrix, with conditions optimized for the accumulation of *m/z* 1500. (A) Spectrum of a small piece of anterior midgut caecum (AMC) tissue, showing a peak at *m/z* 1381.74 corresponding to GYRKPPFNGSIFamide (Gly^1-SIFamide). (B) Spectrum from a small piece of posterior midgut caecum (PMC), showing an intense peak appearing at *m/z* 934.49 identified as APSGFLGMRamide (CabTRP Ia); no signal for Gly^1-SIFamide was detected. (C) Spectrum of an AMC tissue extract, representing peptides extracted from a larger piece of tissue, again showing a peak at *m/z* 1381.74 corresponding to GYRKPPFNGSIFamide (Gly^1-SIFamide). (D) Spectrum of a PMC tissue extract, representing peptides extracted from a larger piece of tissue, again showing a peak at *m/z* 934.49 corresponding to CabTRP 1a; no peak for Gly^1-SIFamide was detected. (E) MS/MS spectrum showing product ions produced by the isolation and dissociation of *m/z* 1381.74 from the AMC tissue extract, providing confirmation of peptide identity via the production of b-type ions (containing the N-terminus) and y-type ions (containing the C-terminus) in a fragmentation pattern characteristic of Gly^1-SIFamide.

4.4. SIFamide likely serves as a midgut-derived hormonal modulator of the Cancer heart

Decapod central pattern generator-effector systems are modulated by both locally released and circulating peptides (*e.g.*, Christie et al., 2010; Christie, 2011). Sources of circulating peptides include neuroendocrine release sites (*e.g.*, Christie, 2011), for example the XO-SG and PO, as well as non-neural tissues, *e.g.*, epithelial endocrine cells present in the midgut (*e.g.*, Christie et al., 2007). With respect to SIFamide and the cardiac neuromuscular systems of *Cancer* crabs, it appears that modulation by locally released peptide is unlikely, as no consistent SIFamide immunoreactivity was found in the CG of either *C. borealis* or *C. irroratus*. Similarly, it seems unlikely that SIFamide modulation of the *Cancer* heart is due to peptide release from either of the two major decapod neuroendocrine organs; no SIFamide immunoreactivity was seen in the XO-SG or PO of either *C. borealis* or *C. irroratus*. It thus seems likely that the influence of SIFamide on the cardiac neuromuscular systems of *Cancer* species is hormonal, via release of peptide from epithelial endocrine cells located in the anterior portion of the midgut/AMC, where authentic Gly^1-SIFamide has been identified in all species thus far investigated, including both *C. borealis* (Christie et al., 2007) and *C. irroratus* (this study).

Essentially all animals exhibit an increase in metabolic rate during the ingestion, digestion, absorption, and assimilation of a meal (*e.g.*, Secor et al., 2007; Mata, 2010; McGaw and Twitchit, 2012; Crocker-Buta and Secor, 2014; McGaw and Penney, 2014; Tirsgaard et al., 2015; Braga et al., 2016), a phenomenon commonly termed "specific dynamic action" (*e.g.*, Secor, 2009). To support this increase in metabolism and to help facilitate the distribution/uptake of nutrients, changes in cardiac output/blood flow typically occur concomitantly with food intake and its subsequent processing (*e.g.*, Secor, 2009). In brachyuran crabs, including *Cancer* species, heart rate and total cardiac output typically increase significantly in response to feeding and remain at elevated levels during the digestive process (*e.g.,* McGaw, 2005, 2006). In at least some species, food can reach the midgut in as little as an hour (*e.g.,* McGaw and Reiber, 2000). If the presence of food in the midgut is a trigger for the release of SIFamide from epithelial endocrine cells in anterior midgut/AMC, then it is possible that the released peptide may be at least partially responsible for regulating cardiac activity during the process of digestion.

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