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ORIGINAL PAPER

Modulatory effects on *Drosophila* larva hearts: room temperature, acute and chronic cold stress

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Abstract Ectothermic animals are susceptible to temperature changes such as cold shock with seasons. To survive through a cold shock or season, ectotherms have developed unique strategies. Our interest is focusing on the modulation of physiological functions during cold shock and prolonged cold exposure in the fruit fly. We use *Drosophila melanogaster* as a model system to investigate cardiac function in response to modulators (5-HT—serotonin, Ach—acetylcholine, OA—octopamine, DA—dopamine and a cocktail of modulators) in acute cold shock and chronic cold shock conditions. Semi-intact larvae are used to provide direct access to the modulators of known concentration in a defined saline. The results show that 10 µM 5HT is the only modulator which maintains heart rate for larva raised at 21 °C and then exposed to acute cold shock (10 °C). The modulators 1 µM OA, 10 µM 5HT, 1 mM Ach, 10 µM Ach and a cocktail of modulators (at 10 µM) increased the heart rate significantly in larvae which were cold conditioned (10 °C for 10 days). HPLC analysis indicated both OA and 5-HT decreased in chronic cold conditioning. The larvae maintain heart function in the cold which may be contributed by low circulating levels of modulators. The larval heart responds better to 5-HT, OA, and Ach in conditioned cold than for acute cold, suggesting some acclimation to cold.

Keywords Serotonin · Dopamine · Acetylcholine · Octopamine · Cocktail · Heart rate · Cold shock · Drosophila · Larvae

Introduction

The unique ability of many invertebrates to survive during environmental temperature changes are a result of various behavioral and physiological strategies, such as burrowing for physical protection, changing the osmolarity of the hemolymph, altering expression of heat or cold shock proteins and production of antifreeze proteins for cold exposure. An index for adaptability to cold in insects is how well they can tolerate cold. For example, a measure which indicates at what temperature there is no longer a response to stimuli or when a loss of motor unit function occurs. At such a point when physiological function stops is termed the critical thermal minimum (Andersen et al. 2015). This measure was recently used for various species of *Drosophila* to determine how well they were able to handle cold stress (Andersen et al. 2015). Some insects, like the ghost moth, apply a strategy to compensate by increasing their metabolism rather than decreasing metabolism (Zhu et al. 2016b). This enhanced metabolism could potentially indicate an increased production of neuromodulators/modulators during long-term cold exposure. However, few studies report on regulation of biogenic amines, peptides, or neuromodulators/modulators in cold acclimated organisms in relation to physiological conditions. Investigating the heritability of heart rate in different strains of *Drosophila* may help to point to particular physiological factors to explain the underlying mechanism (Jennings et al. 2009). Andersen et al. (2015) examined various *Drosophila* species which evolved in different temperate and tropical regions and

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had shown the temperate species had a lower critical thermal minimum than tropical species. To better understand the physiological mechanisms to respond to such stress, genomic and metabolomics approaches are being readily implemented (Colinet et al. 2012). *Drosophila* species offer experimental advantages but they are also limited in addressing extreme cold or prolonged freezing acclimation as they have not been shown to survive freezing conditions (Chen and Walker 1994). However, the ability of some species of *Drosophila* to survive short bouts of cold likely contributes to the wide ecological distribution and numerous species present worldwide.

Repeating a cold exposure with *D. melanogaster* has been shown to enhance survival. This rapid cold-hardening (RCH) ability suggests an expression of cryoprotectant substances and/or physiological changes in the animal. However, Hsp70, glycerol or carbohydrate cryoprotectants were not shown to increase RCH for *D. melanogaster* while other insects did show significant increases in cryoprotectants with cold shock (Kelty and Lee 1999, 2001). Repeated cold exposure reduces apoptotic occurrences in *Drosophila* suggesting cellular protection by reducing the triggers for apoptosis as a potential mechanism for RCH (Yi et al. 2007). RCH treatment of *D. melanogaster* does produce elevated levels (50–100 % increase) of glucose and trehalose. This increase in the osmolality of the hemolymph reduces freezing in the whole animal and likely accounts for prolonging survival in freezing conditions (Overgaard et al. 2007). Slight changes in the amino acid levels are also observed but may not account for survival with RCH (Overgaard et al. 2007). As mentioned in Overgaard et al. (2007), subtle changes in various cryoprotectants, osmolality and modifications in the bi-lipid membrane structure together are the major factors for conditioning (Overgaard et al. 2005, 2006; Lee et al. 2006; Michaud and Denlinger 2006) as these approaches are common for various invertebrate species for cold and dehydration exposure (Burton et al. 1988; Goto and Kimura 1998; Feder and Hofmann 1999; Bennett et al. 1997; Bayley and Holmstrup 1999; Bayley et al. 2001; Overgaard et al. 2007; Tomcala et al. 2006).

Little attention has been given to neuromodulators/modulators with cold exposure in invertebrates. Modulators, such as serotonin (5HT), octopamine (OA), dopamine (DA), and acetylcholine (Ach) as well as peptides, can rapidly alter physiological function in slowing or enhancing physiological processes depending on the target tissue in most all invertebrates investigated. Very small changes in these neuromodulators within synaptic clefts at sites of neuronal communication can have substantial effects on the neuronal function and thus alter behavior and release of hormones possibly involved with cold acclimation. Small changes in the level of released modulators within the CNS of small organisms, such as *Drosophila* larvae, are hard to distinguish. Examining excised brains for analysis does not allow one to know the minuscule released amounts within synaptic clefts as compared to the whole brain. However, modulators released into the hemolymph for distribution within the whole body are feasible, considering one can readily pool hemolymph samples. Modulators such as 5-HT, OA, and DA are well established within crustaceans and insects as being key compounds altering neuronal, cardiac, GI, ventilatory, and skeletal muscle function (review by Shuranova et al. 2006; Strawn et al. 2000; Listerman et al. 2000; Cooper et al. 2011a; b). It is known that DA, 5-HT and OA rise in the hemolymph during exercise in crabs (Sneddon et al. 2000). OA is also known to increase in insects with locomotive activity (Goosey and Candy 1980; David and Coulon 1985). These modulators are generally thought to serve as a stress response in invertebrates (Pagé et al. 2007; Even et al. 2012). OA is known to increase in the hemolymph of locust with heat stress (Davenport and Evans 1984) and there is suggestive evidence that 5-HT may as well increase with heat in locust; however, direct 5-HT measures in hemolymph are lacking (Newman et al. 2003). OA and the precursors for DA and 5-HT all raise in concentration with heat and vibratory stress in the American cockroach (Hirashima and Eto 1993). Even though El-Kholy et al. (2015) did not identify mRNA expression for OA receptors in larval heart of *D. melanogaster* by RT-PCR we still examined the possibility of action by OA on the larval hearts.

To initiate studies on rapid and long-term acclimation to cold and the potential role of modulators in *D. melanogaster* we choose to use a bioindex of cardiac function. *D. melanogaster* utilizes an open vascular system. The heart is a dorsal vessel with the anterior aspect of the tube serving as an aorta. The larval heart of *D. melanogaster* is myogenic and bathed in hemolymph; thus, any modulator can have direct effect on the entire heart (Rizki 1978; Dowse et al. 1995; Gu and Singh 1995; Johnson et al. 1998). The larval *D. melanogaster* heart has been used to investigate the electrophysiological properties of cardiomyocytes in normal and mutant larvae by recording cardiomyocyte action potentials (Lalevee et al. 2006; Desai-Shah et al. 2010) and is a good model to study the role of ions in generation of heart beat (Johnson et al. 1998; Desai-Shah et al. 2010) as well as the effects of modulators (i.e., 5-HT, DA, Ach). With the advent of a recently modified saline which maintains exposed heart for hours, (de Castro et al. 2014) it is now possible to advance to more detailed electrophysiological and cellular studies in the mechanisms regulated the ionotropic and chronotropic actions in the larval heart of this model organism as well as posing questions on the role of modulators during environmental stressors.

Pharmacological and genetic studies in identifying receptor subtypes on the larval heart have revealed putative 5-HT2 (Johnson et al. 1997, 2002; Majeed et al. 2014), type-1 DA and type-2 DA (Titlow et al. 2013), as well as both nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) (Malloy et al. 2015). All three modulators (DA, 5-HT and Ach) show a dose response effect in altering the heart rate (HR). High doses can decrease HR; however, at 10 μM all three modulators produce increases in HR without any further increases in HR at higher concentrations. Preliminary trials with acute exposure of intact *D. melanogaster* larvae from 21 to 10 °C substantially slowed the HR; however, larvae raised at 10 °C had a higher HR than the acutely exposed larvae. This suggested to us regulation beyond cryoprotectant substances and/or hemolymph osmolality changes in maintaining cardiac function as part of acclimation to the 10 °C. Recently, we have shown that using optogenetics and expression of light sensitive channelrhodopsin protein (ChR2.XXL) that cold conditioned larval hearts are able to substantially increase HR, this implies the cold is not physically restricting the ability of the contractile units from functioning (Zhu et al. 2016a).

The purpose of this study was to investigate the role of modulators, specifically 5-HT, OA, DA and Ach on acute cold exposed and chronically conditioned (10 days) cold (10 °C) larvae. The significance of the findings helps to establish a potential role of these modulators in allowing *D. melanogaster* to function with seasonal changes in the natural environments. In addition, the role of modulators may open novel investigations into the mechanisms of maintaining physiological functions with RCH in other insects and invertebrates with cold exposure.

Materials and methods

Fly rearing and stocks

Wild type *Canton S* (CS) flies were used for HR analyses using the semi-intact method and for intact larvae by the "ant farm technique" (Cooper et al. 2009). In brief, the semi-intact method is performed by pinning the third instar larvae ventral side up on a glass plate and dissected in a droplet of saline (shown as a movie, Cooper et al. 2009). The *Drosophila* heart is very sensitive to pH (Gu and Singh 1995); therefore, the saline is adjusted to pH 7.1 and maintained with the high concentration of buffer as described in de Castro et al. (2014). An illustration of the preparation used can be found in Desai-Shah et al. (2010). The third instar larvae were opened by an incision in the ventral midline and the internal organs were washed aside by saline to expose the intact heart to various solutions.

The ant farm technique is a procedure to record intact freely moving larvae. This technique consists of two glass plates (microscope slides; 75 × 25 mm; J. Melvin Freed Brand) narrowly spaced (1–1.5 mm) apart by a thin layer of larvae food, (e.g., moist corn meal—a modified version of Lewis 1960) so that the larvae are able to be visualized within one plane of focus. Spacers commonly used for gel electrophoreses plates (mini gel Bio-Rad; Life Science Research, Hercules, CA 94547, USA) work very well since they can be purchased with varying thickness for use with 1st, 2nd or 3rd instar larvae. Also an option is to use a solid plastic of a given thickness and cut out the region to use as the crawling space. Slightly tilting the platform at 20°–45° causes the larvae to remain, the majority of the time, with their head pointed downward and their tail containing the spiracles out above the food or within an air passage in the food layer. In this configuration the larvae tend not to crawl throughout the food, but instead they only slightly move around in the 2D plane eating so it is relatively easy to count HR. White light is projected from the underside of the microscope stage with a mirror so that it can be moved accordingly for the best contrast of the heart or the two trachea which move while the heart contracts. A microscope (adjustable zoom 0.67–4.5; World Precision Instrument; Model 501379) is used. A 2X base objective and tube objective 0.5X is used to gain enough spatial resolution and magnification to cover a 1 cm by 0.5 cm rectangle. A mounted camera through a trinocular mount is used (Mintron, MTV; World Precision Instrument) and the HR is counted on a TV screen.

This CS strain has been isogenic in the lab for several years and was originally obtained from Bloomington Fly Stock. To obtain staged larvae, the flies were held at 21 °C in a 12 h light/dark incubator before being tested. All animals were maintained in vials partially filled with a cornmeal-agar-dextrose-yeast medium. The general maintenance is described in Campos-Ortega and Hartenstein (1985).

Pharmacology

Acetylcholine, serotonin hydrochloride [5-hydroxytryptamine (5-HT)], octopamine, dopamine-HCl and the salts for making the saline were purchased from Sigma–Aldrich (St. Louis MO, USA). Fly saline, modified hemolymph-like 3 (HL3) (Stewart et al. 1994) containing: (in mmol/L) 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 1 $CaCl_2$, 5 trehalose, 115 sucrose, 25 *N*,*N*-bis-(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) was used with the following modifications: pH was decreased from 7.2 to 7.1 and BES buffer was increased from 5.0 to 25.0 mmol/L to maintain a stable pH (de Castro et al. 2014).

Heart rate measurement

Because heart performance is very sensitive to pH change, the pH was tightly regulated and adjusted as needed. Modulators were applied at various concentrations as indicated in the Results. The preparations were left for 1 min in saline after dissection, and then heart beats were counted for the following minute. The difference in the HR before and after application of drugs was used to measure the effects of the various compounds or temperature.

Experimental conditions

In this study, cold is referring to 10 °C, and room temperature and warm refers to 21 °C. Larvae raised at 21 °C until the early 2nd instar age and then placed in a 10 °C cold room for 10 days were considered cold conditioned. The larvae did molt to a 3rd instar but they were generally smaller than 3rd instars raised at 21 °C. Larvae raised in room temperature until the 3rd instar for experiments were considered as room temperature conditioned larvae. The instar stages were determined using the assay of mouth hook development (Alpatov 1929; Okada 1963). For room temperature conditioned with acute cold exposure with or without modulators, larvae were dissected and the HR was measured at 21 °C with saline and then exchanged to a 10 °C saline or a cold saline containing various modulators in a cold room. For cold conditioned larvae and cold exposure with or without modulators, larvae were dissected and the HR was measured in a 10 °C cold room immersed in 10 °C saline, then the saline was exchange to cold saline (as a control for exchanging saline) or to a cold saline containing modulators of interest. Whole intact larval experiments were performed with the ant farm techniques and exposing the animals to the various temperatures. A thermal probe was placed in the corn meal containing the larvae within the ant farm apparatus to ascertain the correct temperatures.

The mechanical disturbance and time effects on HR

The mechanical disturbance with exchanging the saline plays an important role in altering HR in a semi-intact open preparation (Majeed et al. 2013). Also, HR will slightly decrease over the time in the modified saline used. To account for the mechanical disturbance with exchanging the bathing medium and duration of time effect, a set of control groups were conducted for the same temperature conditions and time frame by exchanging saline with saline only. In the experimental groups, first the HRs were measured in the temperature the larvae were raised and then the saline was exchanged to the temperature of interest and maintained at that temperature for the duration of the experiment.

Four different temperature conditions were examined: (1) room temperature conditioned larvae exposed to acute cold. (2) Room temperature conditioned larvae exposed to acute cold saline containing one or more modulators. (3) Cold conditioned larvae exposed to cold saline. (4) Cold conditioned larvae exposed to saline containing one or more modulators.

HPLC analysis

The quantification of the 5-HT level in third instar larvae were accomplished through high pressure liquid chromatography (HPLC). The hemolymph was obtained from third instars either raised at 21 °C or raised from 2nd instar for 10 days at 10 °C. The third instar larvae were removed from food, washed with water and dried off. To collect the hemolymph, cuticle was nicked directly above the caudal end of the heart after they were lifted into air and a small hole was cut using fine scissors. The larvae were placed into the 1.5 ml Eppendorf tube containing 80 µl of HPLC solution. The larvae were left to sit for 5 min, vortexed lightly for 2 s and then lightly spun down. The larvae were removed with a fine insect dissection pin. The remaining solution in the tube was immediately frozen and stored at −80 °C until HPLC could be performed. The measures were provided per 50 larvae. Given that a single 3rd instar larvae contains approximately 250 nl of hemolymph, the provided values were divided by 50 and concentration estimates of per larvae were based on the MW of the compound of interest. Example: 1000 ng/ml reported would be 20 ng/ml per larvae and for 5-HT this would be approximately 114 nM/larvae. A commercial facility was used to analyze the samples (Center for Microelectrode Technology CenMeT and Parkinson's Disease Translational Center of Excellence, University of Kentucky Medical Center, Lexington, KY 40536-0298).

Statistical analysis

All data are expressed as mean ± SEM. The rank sum pairwise test was used to compare the difference of HR after exchanging solution with saline containing chemicals or temperature. An ANOVA was used to examine the before and after data as a repeated measures and if the data sets were normally distributed data. A Tukey's test was used as a post hoc test following the ANOVA to compare the relative changes of HRs for all the compounds within an experimental paradigm to determine significant differences. This analysis was performed with SigmaStat software. P of ≤ 0.05 is considered as statistically significant. The number of asterisks are considered as $P \leq 0.05$ (*), $P \leq 0.02$ (**), and $P \leq 0.001$ (***).

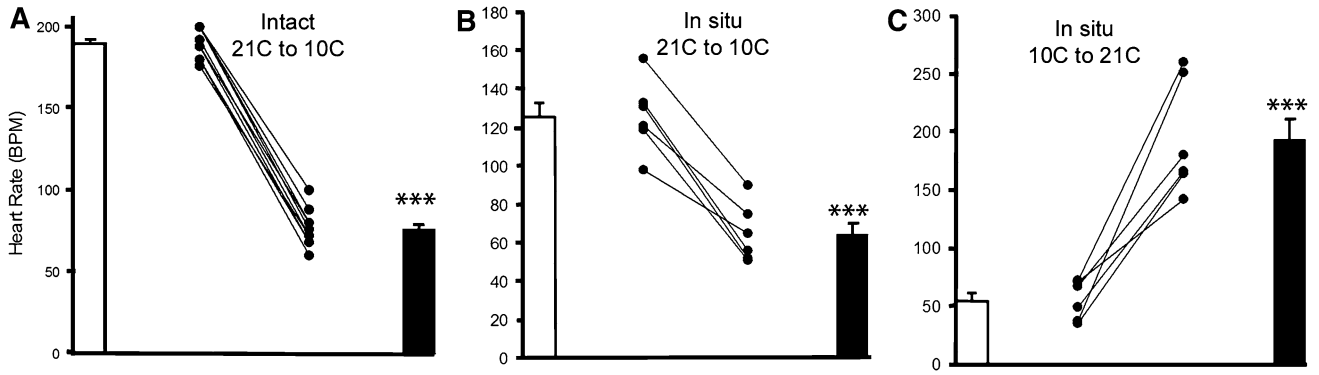

Fig. 1 **a** Change in HR in intact early 3rd instar larvae raised at RT (21 °C) and cold shocked. The heart rate was counted in whole larvae for 1 min and then exposed to 10 °C for 1 min before counting the rate in the next minute. In ten out of ten larvae the HR substantially declined. **b** Change in HR of in situ hearts exposed directly to saline in early 3rd instar larvae raised at RT and cold shocked. HRs were obtained after dissection and exposed to saline at RT and then exposed to saline at 10 °C in a 10 °C cold room. The hearts were allowed to adjust for 1 min prior to counting the rate. **c** The HR of in situ hearts was measured in the cold and then the preparations were removed from the cold room. The saline was exchanged to a saline at 21 °C without any modulators present. $P < 0.001$; rank sum test. The mean (±SEM) of the rates before (*open*) and after (*closed*) the change in temperature are shown as bar graphs

Results

Room temperature conditioned larvae: acute cold exposure

When intact early 3rd instar larvae, which were conditioned to 21 °C, are exposed to rapid cold (10 °C) a drastic decrease in HR occurred. All ten out of ten larvae decreased HR within a minute ($P < 0.001$; rank sum test; Fig. 1a). The average decrease is 60 %. To determine the effect of acute cold exposure on semi-intact dissected larvae, we measured HR before and after exposing cold saline to 21 °C raised larvae. Third stage larvae were dissected at 21 °C and the HR obtained. Afterwards, the preparations were transferred to a cold room and the saline exchanged to cold saline. The HRs during the cold exposure decreased substantially (Fig. 1b; $P < 0.001$; rank sum test). To examine the effect of cold conditioned larvae to warm exposure, embryos were allowed to develop to late 2nd instars at 21 °C and then were placed for 10 days in 10 °C prior to being dissected in the cold. The HR was measured in the cold and then the preparations were removed from the cold room. The saline was exchanged to a saline at 21 °C without any modulators present. In all cases the HR increased sustainably with exposure to the warm saline (Fig. 1c; $P < 0.001$; rank sum test).

Room temperature conditioned larvae: acute cold exposure with modulators

The effect of modulators on HR for 21 °C conditioned larvae were examined at various concentrations (1 μM OA, 10 μM OA, 10 μM 5HT, 10 μM Ach, 10 μM DA, 1 μM DA and a cocktail solution). The modulators were directly exposed to the cardiac tissue when exchanging the bathing media. The effects of the modulators showed varied responses in altering the HR. The acute cold exposure to saline containing OA (10 μM) produced a greater decrease in HR than cold saline on its own (Fig. 2a, $P < 0.05$, non-parametric rank sum). Since some of the preparations completely stopped with 10 μM, a lower concentration of 1 μM was used with the same experimental paradigm (Fig. 2b). Only one of the ten preparations stopped beating with the lower concentration (Fig. 2b, $P < 0.05$, non-parametric rank sum). Also, one preparation showed an increase but nine of ten preparations decreased. However, 10 μM 5-HT exposures for acute cold prevented a decrease in the rate for most preparations (Fig. 2c). Only one preparation had a large drop in HR. Exposure to Ach (10 μM) in the acute cold produced a decrease in HR with two out of the ten having mild increase in HR (Fig. 2d, $P < 0.05$, non-parametric rank sum). Exposure to DA (10 μM) in the acute cold behaved similar to OA with the majority of preparations completely stopping (7 out of 11 preparations) (Fig. 2e, $P < 0.05$, non-parametric rank sum). Since some preparations went into cardiac arrest with 10 μM DA, we also examined if similar effects would occur at a lower concentration. With 1 μM DA, four out of ten preparations stopped while the remaining six preparations showed a decrease to a similar extent as for the 10 μM (Fig. 2f, $P < 0.05$, non-parametric rank sum). We also tested a cocktail of modulators where we generally knew the effect at a given concentration. We used a cocktail of cold solution with each modulator at

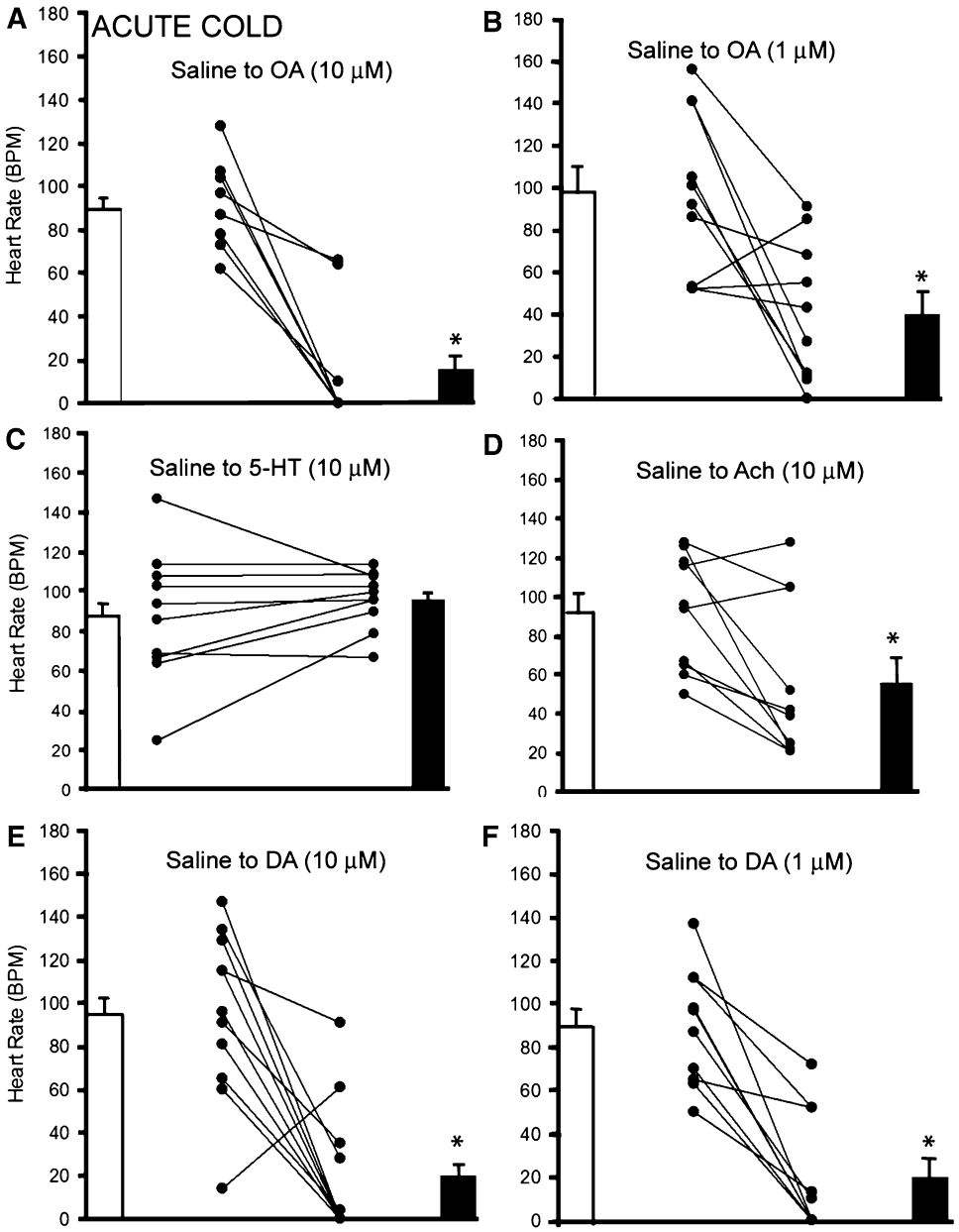

Fig. 2 Change in HR of in situ hearts exposed directly to cold and octopamine (OA). The 3rd instar larvae were dissected and exposed to saline at RT followed by a saline exchange containing OA exchange in a 10 °C cold room. The hearts were allowed to adjust for 1 min prior to counting the rate. **a** In eight out of eight larvae, the HR substantially declined for the 10 µM OA and eight out of ten declined for the 1 µM OA (**b**). **c** Saline (21 °C) exchanged to one containing 5-HT in a 10 °C cold room resulted in four out of ten larvae to increase and two out of ten larvae to decrease while four out of the ten did not show substantial change for the 10 µM 5-HT. **d** Saline exchange to cold saline containing Ach (10 µM) resulted in eight out of ten larvae to decrease HR. **e** Saline exchange to cold saline containing DA (10 µM) resulted in 9 out of 11 larvae to substantially decrease and 9 out of 10 to decline for exposure to 1 µM DA (**f**). The mean (±SEM) of the rates before (*open*) and after (*closed*) the change in temperature and exposure to the compounds are shown as bar graphs

10 µM (OA, DA, Ach, and 5-HT). All ten larvae showed a decrease in HR and two completely stopped (Fig. 3a). The acute cold cocktail had a significant change in decreasing HR ($P < 0.05$, non-parametric rank sum). Since preparations showed a large variation in initial HRs, a percent change from 21 °C to cold or cold and modulators, were compared (Fig. 3b). The only group different from cold saline exposure itself is the 10 µM 5-HT since the rates increased instead of decreasing. The parametric statistical analysis did not indicate differences from exchanging saline alone to the other modulators as the data are not normally distributed with HR stopping for a number of preparations with DA and OA.

Cold conditioned larvae: influence of modulators

The cold conditioned larvae showed no effect of exchanging the saline with cold saline to account for mechanical disturbance. Such saline disturbance does alter HR for larvae maintained at 21 °C (de Castro et al. 2014, Fig. 2; Majeed et al. 2013, Fig. 1; 2014, Fig. 1). There is a significant difference in the effect of a bath change on HR with room temperature conditioned larvae on acute cold exposure as compared to saline exchange in cold conditioned larvae with a cold saline exchange ($P < 0.05$, Mann–Whitney; Fig. 4a).

Modulators effect on cold conditioned larvae was also tested at different concentrations (10 µM OA, 1 µM

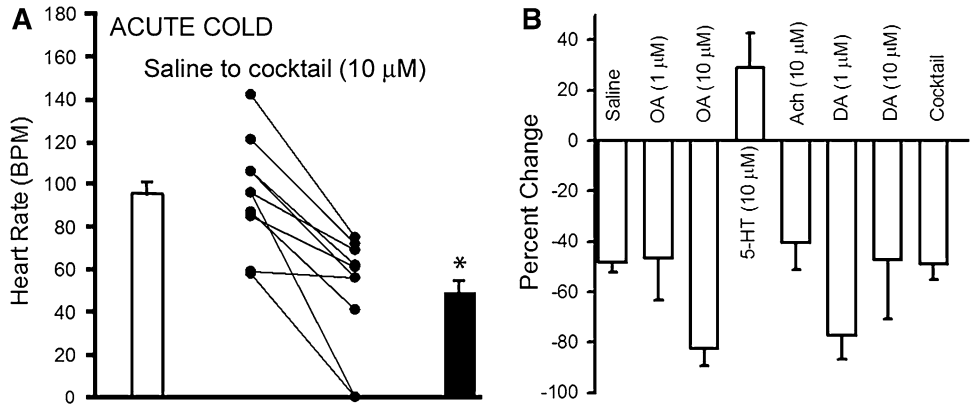

Fig. 3 Change in HR of in situ hearts exposed directly to cold and a cocktail of modulators each at 10 μM octopamine (OA), serotonin (5-HT), dopamine (DA) and acetylcholine (Ach). Larvae were raised at 21 °C. The 3rd instar larvae were dissected and exposed to saline at RT. This was followed by an exchange to cold saline containing the modulator cocktail in a 10 °C cold room (**a**). A comparison in the percent change of each individual modulator and the cocktail for the acute cold shock is shown in (**b**) for comparison. The percent change in rate was determined for each individual larvae and then averaged (mean ± SEM)

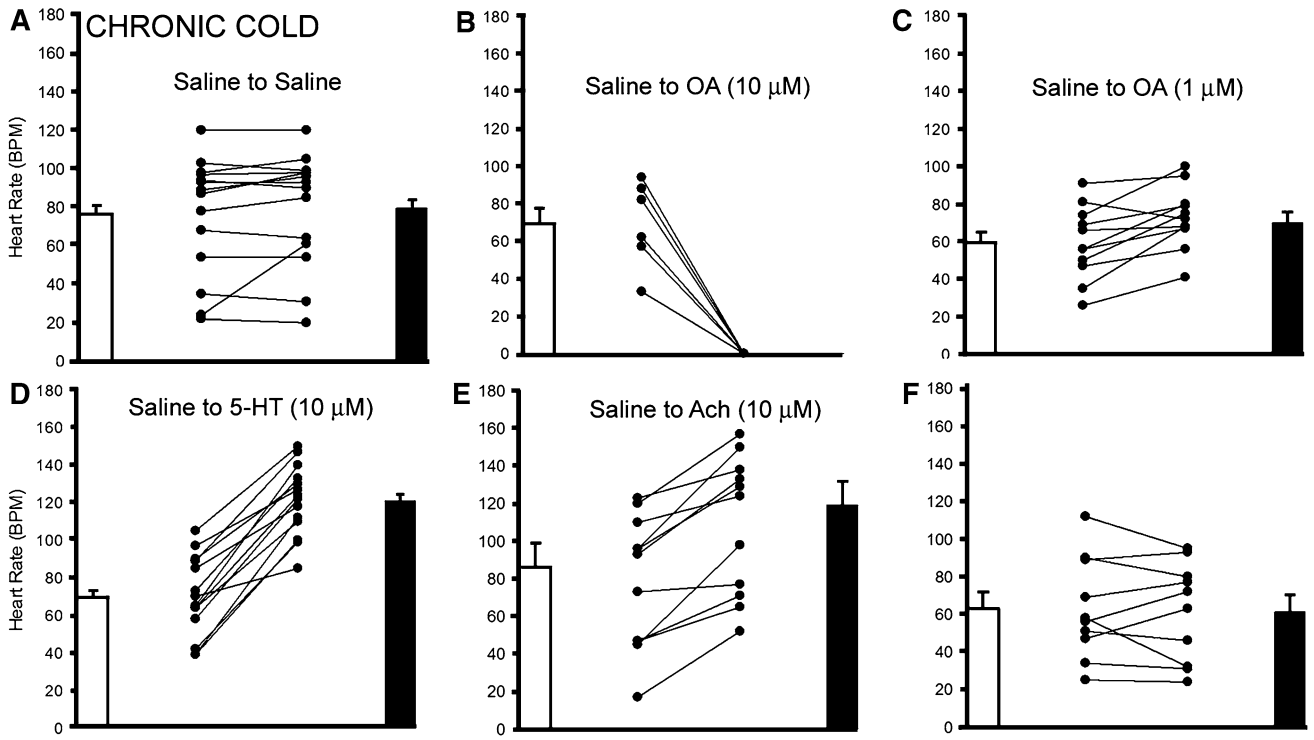

Fig. 4 Change in HR of in situ hearts in cold conditioned larvae. Larvae were raised at 10 °C from 2nd instar for 10 days and then dissected in early 3rd instar stage. HRs were obtained after 1 min following the dissection which occurred in a 10 °C cold room. The hearts were allowed to adjust for 1 min prior to counting the rate after saline only exchange or with saline containing a modulator. The saline to saline only exchange was to control for any movement effects on the heart by exchanging the saline. No overall significant differences occurred for exchanging the saline (**a**). One out of 14 larvae showed an increase. **b** Exposure to octopamine (OA) for cold conditioned larvae resulted in six out of six larvae substantially decrease HR and even stop during a 10 μM but 10 out of 11 larval HRs substantially increased with a 1 μM OA exposure (**c**). **d** Cold conditioned hearts exposed to serotonin (5-HT, 10 μM) resulted in 14 out of 14 larvae to substantially increase HR. **e** Hearts exposed to acetylcholine (Ach, 10 μM) resulted in 11 out of 11 to substantially increase in rate. **f** Dopamine (DA, 10 μM) exposure resulted in six out of ten larvae to increase their HR

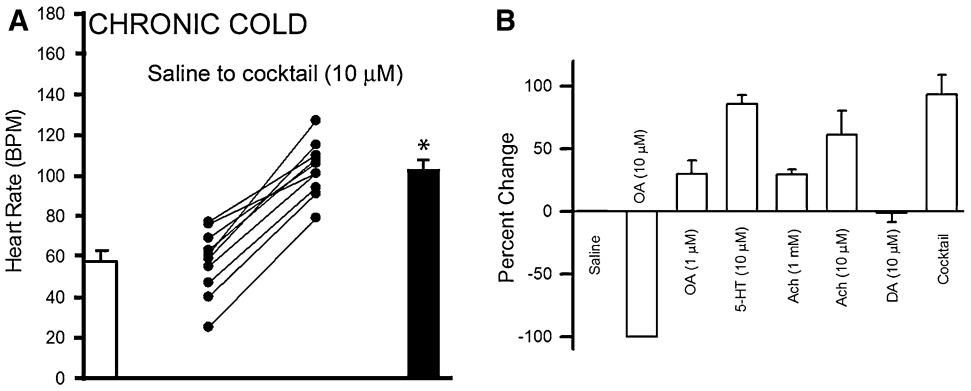

Fig. 5 Change in HR of cold conditioned larvae where the in situ hearts are exposed directly to a cocktail of modulators each at 10 µM octopamine (OA), serotonin (5-HT), dopamine (DA) and acetylcholine (Ach). All ten out of ten larvae substantially increased their HR (**a**). A comparison in the percent change of each individual modulator and the cocktail for the chronic cold exposure larvae is shown in **b** for comparison. The percent change in rate was determined for each individual larvae and then averaged (mean ± SEM)

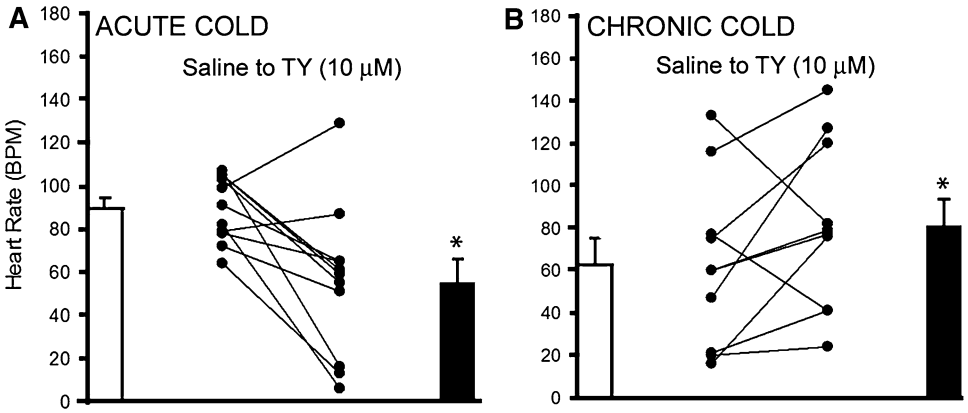

Fig. 6 The effect of tyramine on acute cold shock and cold conditioned hearts. **a** Tyramine at 10 µM on acute cold exposure hearts produced a decrease in HR even more so than cold saline exposure alone. **b** Tyramine on cold conditioned hearts produced for the most part an increase in HR (eight out of ten preparations)

OA, 10 µM 5-HT, 10 µM DA and cocktail solution). In all six preparations examined, OA at 10 µM stopped the heart (Fig. 4b, $P < 0.05$, non-parametric rank sum). With a reduced concentration of OA (1 µM) only 1 preparation out of 11 showed a decrease with most having a mild increase in HR (Fig. 4c, $P < 0.05$, non-parametric rank sum). However, 5-HT at 10 µM produced substantial increases in HR with cold conditioning (Fig. 4d, $P < 0.05$, non-parametric rank sum) as well as Ach at 10 µM (Fig. 4e, $P < 0.05$, non-parametric rank sum). DA was not as consistent in its effect at 10 µM with six out of ten preparations increasing HR and decreasing in others (Fig. 4f). As for the RT to acute cold exposure, a cocktail of the modulators (OA, DA, Ach and 5-HT, each at 10 µM) was examined for the cold conditioned larvae. In all cases, ten out of ten preparations, there was a substantial increase in HR (Fig. 5a, $P < 0.05$, non-parametric rank sum). Since there is a large variation in initial HRs a percent change normalizes the trends for ease in comparisons to the effect of the modulators. It is readily apparent 10 µM OA showing an opposite effects from the other modulators at the same concentration. The exchange of saline on cold conditioned larvae did not produce any notable effect (Fig. 5b). In comparing the percent changes for the various modulators, 1 µM for OA and 1 mM for Ach is also shown in the graph for comparisons. The greatest increases in HR are with 5-HT and the cocktail. It is interesting to note the inhibitory effect of OA at 10 µM was overridden with the cocktail containing the other modulators. Even the negligible effect of DA did not appear to dampen the effect of the cocktail.

A recent publication by (El-Kholy et al. 2015) appeared in which OA receptors were not able to be identified in larval heart by RT-PCR utilizing receptor gene specific primer pair. However, a tyramine receptor (TyrR) was shown to be expressed in the heart. So we also assayed tyramine at 10 µM for acute cold and cold conditioned larvae. In 9 of 11 preparations, HR decreased with acute cold exposure ($P < 0.05$, non-parametric rank sum) and 8 of 10 preparations HR increased in cold conditioned larvae (Fig. 6a, b). The percent change for acute cold is -39 ± 12 % and the percent change for cold conditioned is 72 ± 39 %.

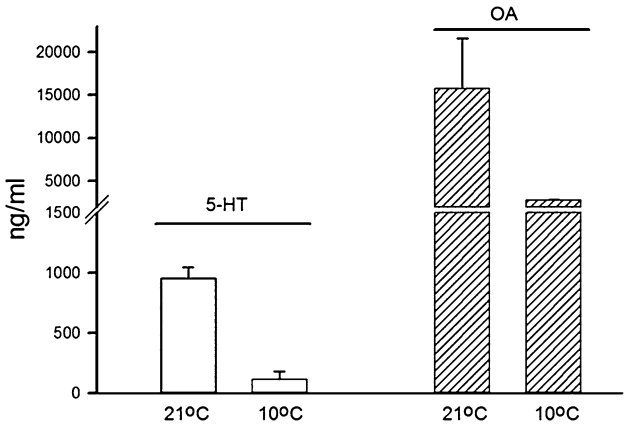

Fig. 7 Analysis in the changes in the concentrations of 5-HT and OA within the hemolymph of larvae raised at 21 °C and larvae conditioned to cold (10 °C) for 10 days. Each sample contained pooled hemolymph from 50 larvae and three samples in each conditioned were analyzed (mean ± SEM)

HPLC analysis of hemolymph

The quantification of OA and 5-HT in 3rd instar larva hemolymph showed different concentrations by HPLC analysis. The average value of 5-HT in room temperature raised larva is 957 ng/ml or 109 nM per larva based on three samples. Meanwhile, the 5-HT level of larvae raised in the 10 °C decreased to 116 ng/ml or 13 nM. The overall OA level is substantially higher than the 5-HT, and OA value is decreased from 21 °C raised larva 15,719 ng/ml or 2052 nM to cold conditioned larva 2808 ng/ml or 367 nM. To convert these values to concentration per larva, the volume of hemolymph was measured to approximately 250 nl. The results indicated a drop in the circulating levels of OA and 5-HT over 10 days of conditioning in the cold as compared to larvae maintained at 21 °C (Fig. 7).

Discussion

In this study, it was demonstrated that acute and chronic exposure to cold (21–10 °C) reduced the HR in larval *D. melanogaster*. However, when the hearts were directly exposed to 5-HT upon acute or chronic cold exposure the HRs showed a substantial increase. Other known modulators (DA, Ach and OA) did not have a pronounced effect on reducing the cold response on HR; however, at room temperature all four modulators combined (5-HT, DA, Ach and OA) have a significant effect in increasing HR (Malloy et al. 2015; Majeed et al. 2014; Titlow et al. 2013; Zornik et al. 1999). In addition, OA alone or DA alone at 10 μM produces marked increases in HR at room temperature (Majeed et al. 2014; Titlow et al. 2013); however, at 10 °C these compounds produce a cessation of HR in a number of trials. Lower concentration of OA or DA (1 μM) did not cause the cold hearts to stop. The HPLC results show a decrease in OA in cold conditions which may in fact help to keep the hearts functioning if higher concentrations reduce the rate. These studies indicate a differential degree in the responsiveness to modulators in altering HR to acute and chronic cold exposure. Addressing the potential effects of modulators in offsetting the physical effect of cold shock and longer term cold exposure are novel results as well as the effects of a modulator cocktail. It appears that effects of the modulators are not additive when combined in a cocktail.

The rationale to focus on these four modulators specifically is that these are commonly investigated to assess modulation on neuronal and heart function in insects and crustaceans. In addition, recent pharmacological and genetic manipulations have putatively identified the receptor subtypes for dopamine as type-1 DA and type-2 DA (Titlow et al. 2013), and for serotonin 5-HT2 receptors as the main subtype (Majeed et al. 2014) on the larval heart. There, appears to be both nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs) (Malloy et al. 2015) on the larval heart and in this study, we present data that the larval hearts are sensitive to octopamine when provided directly to the heart in a defined saline.

A previous study had shown that larval hearts are not sensitive to octopamine at concentrations less than 10 μM. However, this study was performed by injection into the hemolymph of the whole animal while restrained (Zornik et al. 1999). A recent study examining the expression profiles of OA and tyramine receptors only found TyrRs present in the larval heart and not OA receptors (El-Kholy et al. 2015). So, the responses we observed in acute exposures to cold and cold conditioned flies to OA 10 μM warrants discussion. Zornik et al. (1999) did report at 100 μM an 86 % increase over a baseline HR in larvae and responses to lower levels in injected pupa for OA. Raw data are not presented in this earlier study; thus, we cannot know what the rate was changing from with the saline injections used to obtain the percent changes for the modulators. We predict the injection and restraint of the larvae likely induces some stress where modulators may already be released into the hemolymph. We report baseline values for the effect of the saline to saline exchanges to compare to the effects of mechanical disturbances on the hearts. It was reported that just exchanging the bathing fluid over the heart will alter HR with usually a 10–15 % increase in the rate (Becnel et al. 2013; Majeed et al. 2014; Titlow et al. 2013; de Castro et al. 2014). On the other hand, rapidly dissecting the larvae and washing out the hemolymph to expose the heart to a defined salt solution is not as natural

as in vivo preparations, but it does allow multiple variables to be reduced to defined conditions for comparison. Since we know 5-HT, OA and DA, all work on the larval CNS to excite neural circuits and enhance motor unit activity; this could have substantial effects in intact larvae for hormonal release and even contracted and relaxed states of body wall muscles (Dasari and Cooper 2004). As for the lack in expression of OA receptors on the larval heart, it does appear that there is little if any GFP expressed in the transgenic lines in which the presumptive promoter for all OA receptors is utilized (El-Kholy et al. 2015). Since tyramine receptor expression is observed in the larval heart, potentially OA is activating tyramine receptors and initiating the effects we observed. OA and tyramine are similar in chemical structure as tyramine is a precursor to OA (El-Kholy et al. 2015).The interesting point is that OA at 10 μM causes the heart to stop upon exposure in the cold; whereas, the lower concentration did not show as prominent response any different than just a saline exchange for the acute cold saline exposure. Using the same concentration of tyramine and OA (10 μM), OA showed a larger degree of change than tyramine. Both compounds decreased HR in acute cold exposure. If OA and tyramine were binding to the same receptors it would appear OA produced a greater response. Since OA is also similar in chemical structure to DA, it may also be possible that OA is binding to DA receptors and inducing a response. However, OA actions are greater than that shown for DA at the same concentration for acute cold. Only OA, DA, and tyramine at 10 μM showed a strong trend to decrease HR with acute cold, whereas Ach and 5-HT did not. However, Ach and 5-HT did reduce the inhibitory effect of the cold exposure. So, even if OA is activating specific OA or tyramine receptors on the heart, the mechanism responsible for stopping the heart is not known. Note that at warmer temperatures (21 °C) OA and DA increases HR within the defined saline. Since OA receptors are G-coupled, may be the Gαq subunit is activated but in cold this action is suppressed and Gαi-coupled receptors are activated. This remains to be examined with further pharmacological studies by blocking the potential Gαq activation of phospholipase Cβ (PLC) with OA at 21 °C like it was performed in addressing the mechanism of 5-HT's action on the heart (Johnson et al. 2002; Becnel et al. 2011, 2013; Majeed et al. 2014) and suppress Gαi in cold conditioned or acutely exposed hearts.

Addressing the many potential biogenic amines, catecholamines, peptides and other substances known to be in insect and crustacean hemolymph which alter cardiac function in a one by one fashion will take a long time and may not be informative enough of the true physiology in the whole animal but at the same time one is stuck with compounding indirect effects by addressing these actions within the animal. As Marder (2012) pointed out in a review of modulation in neural circuits in invertebrates, it is highly unlikely any one modulator is working in isolation. This is likely true for the cardiac function for insect hearts as well. At least in the early larval stages the heart is devoid of direct neural innervation (Johnstone and Cooper 2006); thus, in the isolated in situ early 3rd instar studies with bathing media of known composition being exchanged, the indirect effects of any substances being released by stimulating neurohumoral actions is minimized. Cocktails of modulators in known quantities can be examined for their effects on the heart to compare to actions of individual modulators as we have approached in this study. The interesting finding here is that 5-HT appears to be the dominate modulator in reducing the effect of cold shock and long-term cold conditioning by itself. Also, as a cocktail the excitatory effect of 5-HT overcame the inhibitory action caused by DA and OA at 10 μM for acute cold exposure. The timing in exposing tissue to individual modulators when applying them in a sequential series can be problematic as noted in a crustacean study of modulation of synaptic transmission at the neuromuscular junction in applying OA and then 5-HT as compared to 5-HT then OA (Djokaj et al. 2001). As noted, there are numerous experimental paradigms to mimic in vivo exposure of modulators with still keeping track of the introduced variables.

The results of the HPLC revealed that cold conditioned, decreased the level of OA and 5-HT drastically in the hemolymph. The reduction in 5-HT and OA was counterintuitive in view that 5-HT increased HR at 21 °C. The reduction may well be due to a reduced production or release as compared to warmer conditions. As the modulators turnover, there may be a reduction in biochemical syntheses and release into the hemolymph in the cold resulting in the lower levels measured. It is unlikely that an increase in the degradation is taking place in the cold since overall metabolism is likely decreased. Potentially even receptor expression levels could be altered. However, to address these specific points, more detailed experimentation is required. Measures in other invertebrates in non-stressful temperatures via HPLC revealed for the honey bee is about 17.5 nM 5-HT (French et al. 2014), whereas the 5-HT concentration in tobacco cutworm is in the range of 15–25 nM (Ikemoto et al. 1993). In *Aplysia* OA is around 100 nM (Levenson et al. 1999). It would be of interest for comparison if future studies would address the changes in the levels of modulators with acute and chronic cold conditions.

The different magnitude of action in altering HR for the modulators during acute cold as compared to chronic cold (10 days) warrant further studies in the potential mechanisms. The cold stress is likely to cause many physiological responses which would account for the differences; however, a likely scenario is the altered levels of endogenous modulators and/or altered expression of receptors as well

as even differential expression in the subtypes for a given modulator. The paradigm we used of rapid cold shock and maintenance at 10 °C prior to examining the effects of modulators might not have allowed the same physiological responses as those responsible for RCH. If we gradually reduced the temperature or pulsed the cold shock a few times prior to holding the larva in the cold, the pulsing of cold shocks in a gradual manner may allow the animal to have periods of higher metabolism to conduct physiological alterations in preparation for the cold as noted in other studies on survival with cold exposure (Czajka and Lee 1990). It would also be of interest to examine the effects of diet in natural habitats for insects in response to cold shock which as far as we are aware has not been addressed fully. This could be examined well in laboratory conditions and even the responsiveness to modulators for larvae or adults cultured in various media. Differences in diets in the natural environment might also account for survival among insect species to cold exposure. It is known different *Drosophila* species are noted for varying abilities to acclimatize to cold (Graham et al. 2012; Vesala et al. 2012). What cellular responses might be driving this ability is the key to understanding the mechanisms. The effect of temperature on HR with exposure to deuterium oxide revealed that exposure to deuterium oxide reduced the changes in HR with temperature (White et al. 1992). The mechanism by which this occurs is still unknown. Recovering from cold upon re-exposure to higher temperatures may likely involve regulation of endocrine factors (Terhzaz et al. 2015). There are a number of remaining studies needing to be addressed in relation to cardiac function in *Drosophila*. Identifying hormonal changes in the hemolymph with various environmental conditions and repetitive exposures may explain selective acclimation. It would be interesting to take the hemolymph of cold conditioned larvae and apply to acute cold exposed hearts to determine if HR is increased or not. In addition, examining the irregularity in beats would be good to assess over time for a better representation of the effects of various conditions (Jennings et al. 2009). The rates over the time counted in this study did vary depending on the condition; thus only average rates are reported.

A recent study on crayfish *(Procambarus clarkii)* and prawn *(Macrobrachium rosenbergii*), which both are native to warm waters, showed marked difference in behavior and cardiac physiology when exposed to cold abruptly as well as in a gradual manner over weeks (Chung et al. 2012). With an acute temperature change (21–5 °C) the prawns died within 2 h; whereas, crayfish were still alive and responsive to touch on their telson for days. Monitoring the HR indicated the prawns stopped any alteration in the HR in conjunction with the lack of notable behavioral changes to stimuli; whereas, crayfish which only showed slight behavioral responses still produced significant alteration in HR when physically disturbed. Such hardiness in this species of crayfish may well account for their wide distribution in North America and invasiveness after being introduced in Europe and Asia (Ackefors 1999; Nyström 1999; García-Arberas et al. 2009).

With knowing that epigenetic factors are tied to environmental stressors and that RCH is more pronounced with repetitive exposures, it would also be of interest if there is a correlation to account for selective protein expression not only within a species but also among *Drosophila* species exposed to the same stressors (Vesala et al. 2012). In addition, being able to pin point potential receptors or cellular processes which may signal cold and produce hormonal changes as a response is a target of interest to potentially understand the cascade of events to cold conditioning. Interestingly, some HSP increase with cold and heat exposure, and may serve the same function in stability of particular proteins. It is of interest to note that changing cold saline with cold saline in cold conditioned larvae did not produce an increase in HR as known to occur with larvae raised at 21 °C and exchanged with 21 °C saline (Majeed et al. 2014; Titlow et al. 2013, de Castro et al. 2014; Malloy et al. 2015). The small increase in HR at 21 °C with exchanging the saline is likely a response due to stimulating stretch-activated ion channels known to be on the heart in mammals (Baumgartner et al. 2012) and are present in *Drosophila* sensory neurons (Coste et al. 2012). Considering the increase in HR does not occur with cold conditioned larvae when the saline is exchanged could indicate the channels are too rigid in the cold and that the possible accessory proteins may not be maintaining the structural integrity of the channel to sense the mechanical movements. It may also be possible that the cold conditioning has activated the expression of chaperonin HSP which stabilize the membrane and prevent mechanical deformation and dampen the effect of stretch-activated channels (Kayukawa and Ishikawa 2009). We are now addressing these possibilities in a follow up study (abstract, de Castro et al. 2015).

While our methods likely do not reflect natural conditions, our data indicate that neuromodulators can affect cardiac function of cold conditioned larvae. Thus, subsequent experiments will focus on testing the in vivo function (RNAi approaches) of these neuromodulators during the course of cold conditioning. Maintaining HR in the cold is essential for circulating nutrients/cryoprotectants, immune function and since we demonstrated that modulators do have activity at low temperature suggests they may be ecologically important.

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