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Species - Dependent Cardiac And Motor Responses To Cholinergic Stimulation In Daphnia Pulex And Daphnia Magna

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SPECIES – DEPENDENT CARDIAC AND MOTOR RESPONSES TO CHOLINERGIC STIMULATION IN DAPHNIA PULEX AND DAPHNIA MAGNA

by

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Dedication

I would like to dedicate this work to the memory of my late mother, Joan Hannan, whom without I would not be who, or where I am today. To my amazing father, Don Hannan, for continuing to push me to strive to be my best, teaching me the meaning of hard work, and always believing in me, even when I didn't. And to my incredible sister, Jaime Hannan, who has been the best academic and life role model a brother could ask for.

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In loving memory of my Mother and Grandpa Buck…

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Preface

All of the work presented was conducted at the Eugene Applebaum College of Pharmaceutical Sciences.

A version of Chapters 1 and 2 are currently being reviewed for publication by the Journal of Comparative Biochemistry and Physiology

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Introduction

Daphnia are small invertebrate crustaceans belonging to the order, Cladocera, and are *ubiquitous,* found in most freshwater ecosystems throughout the world. *Daphnia* are primary grazers of phytoplankton, detritus, and bacteria and are considered to be a keystone species critical to the structure of aquatic food webs (Carpenter et al., 1987; Dodson and Hanazato, 1995; Tessier et al., 2000). There is an extensive literature on *Daphnid* ecology, and they have been used extensively as sentinels for the evaluation of water quality and ecotoxicity (Arndt et al., 2013; Dodson and Hanazato, 1995; Guy et al., 2011; Hannas et al., 2011; Ignace et al., 2011; Lovern et al., 2007; Moosus and Maran, 2011; Niculescu et al., 2008; Rider and LeBlanc, 2005). Two of the species most commonly used for evaluating water quality and chemical toxicity are *Daphnia pulex* and *Daphnia magna*.

Over the past two decades very significant advances have been made in understanding circulatory and ventilatory function in *Daphnia* by the research group at the Institut für Zoophysiologie (Gerke et al., 2011; Goldmann et al., 1999; Paul et al., 1997; Paul et al., 2004; Pirow et al., 2002, 2004; Pirow et al., 1999; Zeis et al., 2013). However, even with these advances, and the continued use of *Daphnia* in evaluating chemical toxicity and setting water quality criteria, there are still many unknowns associated with neurochemical signaling and the control of motor function. A better understanding of the *Daphnid* neurohormonal regulation of motor

function will enable a deeper understanding of the nature of responses to biotic and abiotic influences and enable comparisons across species or phyla.

With the recent sequencing of the genome for *Daphnia* there is increasing interest in the utility of *Daphnia* for genomic research (Christie and McCoole, 2012; Colbourne et al., 2011). The organisms are ideally suited for genomic studies because of their ease of maintenance in a laboratory, small size, short life cycle, large brood size, and asexual reproduction (cloning), and are considered a model organism by the National Institute of Health (www.nih.gov/science/models). Because *Daphnia* are very sensitive to biotic and abiotic changes in their environment, and have developed specific adaptive strategies to cope with changes in temperature, water chemistry (e.g., dissolved oxygen), food supply, and predation, they are useful for toxicogenomic studies and are used as indicator organisms or 'biosensors' (Shaw et al., 2008). The analytical power as a toxicogenomic assay will be enhanced by a foundation of physiological, pharmacological, and toxicological knowledge that addresses the functional attributes associated with the expression of specific genes.

Early pharmacological studies of the *Daphnid* heart can be found in the literature at least as far back as the late 19th century (Pickering, 1894), and early 20th century (Levy, 1927). In 1936, Henry Dale and Otto Loewi shared the Nobel Prize in Physiology or Medicine for "*their discoveries relating to the chemical transmission of nerve impulses*" based on the pioneering work that led to the discovery of the physiological role of acetylcholine in frogs as well as mammalian bodies (ACh)(Tansey, 2006). By mid-20th century a number of pharmacological studies were reported that examined responses of the *Daphnid* heart, appendages or gut to

various pharmacological agents (Baylor, 1942; Bekker and Krijgsman, 1951; Obreshkove, 1941; Oconnor, 1950; Sollmann and Webb, 1941). Many of the pharmacological agents used in these studies target mechanisms associated with the neurotransmitter, ACh. Many of these early studies on *Daphnia* were based on the established important role ACh plays in the parasympathetic nervous system and neuromuscular junction of vertebrates.

Baylor (1942) studied heart function in *D. magna* utilizing a technique that involved trapping an animal in cotton fibers on a depression slide, and applying drug solutions externally with pipettes. The animals were maintained at a temperature of 10^{o} C so that the rapid contractions of the heart could be slowed down sufficiently to count them visually. Baylor found that ACh application in the micromolar range could inhibit the heart rate by about 20%. Baylor also found that concentrations of physostigmine higher than 10^{-8} M (an AChE-I) appeared to inhibit HR, but its actions were deemed toxic and difficult to interpret. Baylor reported that the muscarinic antagonist, atropine, inhibited the heart rate, but the reported inhibition appeared to be less than 20% even with high concentrations (10^{-2} M), and was described as probably a toxic effect. The pharmacological interaction between ACh and atropine was apparently not studied. Responses to epinephrine and potassium chloride were also studied and, ultimately, Baylor concluded that the pharmacology of the *Daphnid* heart resembled the vertebrate heart more than that of higher crustaceans.

Bekker and Krijgsman (1951) expanded Baylor's work by studying *Daphnia* at the same temperature (10^{o} C), but improved the drug administration method. In this study the animals were inserted into a tube that included a two-way stopcock at one

end (for water or drug solution) so that solutions could be applied at a constant flow rate. As in Baylor's experiments (1942), the animals were prevented from moving by using cotton fibers for immobilization. In addition, the study by Bekker and Krijgsman included two different species; *D. magna* and *D. pulex*. The authors claimed that there were no species-dependent differences in the responses of the *Daphnid* hearts in their study. Bekker and Krijgsman found that ACh (~ 100 to 500 nM), the muscarinic agonist, pilocarpine (~ 50 μM), and the AChE-I, tetraethyl pyrophosphate (~ 50 to 100 μM) slowed heart rate, while atropine (~ 50 to 500 μM) increased heart rate. Bekker and Krijgsman concluded that their overall results were consistent with the assumption that *Daphnia* have a myogenic pacemaker that is inhibited by "extra-cardiac cholinergic nerves."

To the best of our knowledge this is the first pharmacological study since Baylor (1942) and Bekker and Krijgsman (1951) to address the regulatory role of the cholinergic system in controlling the *Daphnid* heart (HR) and the appendage beat rate (ABR). Since there is an extensive literature on the ecology of *Daphnia,* a long history of use in water quality testing, and now a fully sequenced genome, this provides a promising zooplankton tool for the analysis of responses to multiple environmental stressors in a very adaptive organism (Colbourne et al., 2011). The intent of this study is to gain better understanding of the role of the cholinergic system in regulating critical motor functions. The hypothesis tested is that the beating of the heart, thoracic appendages and swimming antennae, and the fast motor tremor of the eye are modulated by cholinergic activity. An additional

hypothesis is that there are species-dependent differences in cholinergic regulation of cardiac and motor activity.

Methods

Animals

D. pulex and *D. magna* were housed in separate 4 L jars containing artificial freshwater media, COMBO (Kilham et al., 1998), and housed in an incubator at a temperature of 21 °C, and at a light/dark cycle of 16/8 h using broad-spectrum fluorescence lights (5000K Ultralume). Animals were fed a 50/50 mixture of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardii* algae 3 times per week and approximately 1/3 of the media was refreshed with COMBO media 2 times per week. The jars were cleaned and filled with fresh COMBO media once per week.

Aquatic chamber

A custom built aquatic chamber, manufactured by the College of Engineering machine shop at Wayne State University, was constructed from 1.5 cm thick Plexiglas (Figure 1). The base of the chamber is rectangular in shape, measuring 8.0 cm x 3.2 cm x 1.3 cm (LWH) with a 2 cm diameter cylindrical hole drilled through the middle. A cylindrical Plexiglas chamber-viewing insert with a rubber gasket was used to close off the top of the chamber in the rectangular Plexiglas base. The chamber-viewing insert also had a 7.5 mm diameter hole in the middle for viewing the specimen, and the bottom of the insert was closed with a round 1.5 cm diameter glass coverslip. A standard rectangular borosilicate glass microscope slide was used to close off the bottom of the Plexiglas chamber and create a sealed chamber. Two

23-gauge stainless steel hypodermic tubes were used as the inlet and outlet of the Plexiglas chamber and allowed the chamber to be perfused with solutions. A small open well connected by the steel outlet tube was used to collect the perfusate that exited the chamber. The temperature of the perfusate was monitored just after it exited the sealed chamber with a needle microprobe (Physitemp Instruments, Inc., Clifton, New Jersey), and before it reached the open well where the solution was suctioned away. When the chamber was mounted on the microscope stage for viewing, it rested on top of a Physitemp Instruments, Inc. TS-4SPD heating and cooling stage (Figure 1).

Animal Preparation

Adult female *D. magna* and *D. pulex* were visually selected for the experiments based on size so that only adult animals were included in experiments. Approximate range of animals selected for experiments was 2.8 – 3.4 mm for *D. magna* and 1.4 to 2.3 mm for *D. pulex*. Length was chosen based on measurements of adult stage *Daphnia*. Animals were isolated from the culture jar and placed ventral side down into an appropriately sized rounded groove on a piece of plastic. They were then moved into an ideal gluing position using a small single-hair brush to minimize any damage or stress. With the dorsal side facing up a very small drop of cyanoacrylate glue (<150nl) was placed on the tip of a 33-gauge syringe needle (approximately 10 mm in length). Using forceps, the needle was kept in parallel alignment with the body and glued to the dorsal side of the head shield, slightly anterior to the heart and posterior to the eye. The glue was allowed to dry approximately 10 seconds before moving the animal. The needle holding the animal was then placed into a holder

within the aquatic chamber. The chamber was filled with COMBO media and the top chamber-viewing insert fixed into place. All animals could freely move their swimming antennae and appendages while in the chamber.

Drug perfusion experiments

The chamber was placed on the TS-4SPD heating and cooling stage fixed to a Nikon FN600 light microscope and the animal was viewed at 40x magnification (See Figure 1). A clear view of the animal's moving heart, appendages, swimming antennae, eye and gut was visible through the translucent exoskeleton. The chamber was connected to a BASi hydraulic switch and microliter syringe pump (BASi, West Lafayette, IN). The syringe pump could hold three 2.5 ml gas-tight syringes that could be selected to pump COMBO media through the chamber and the perfusate temperature was controlled by the heating and cooling stage sitting under the glass bottom chamber. Video recordings were taken every 10 min for 10 sec using an Infinity 2M-1 monochrome camera and associated software. Motor activity was analyzed using Image Pro Plus 2-D tracking software (Media-cybernetics, Inc., Rockville, MD). Small-circumscribed areas of interest were identified where motor activity was occurring, and density-intensity changes representing movement of specific muscles were then monitored and counted over time.

Experiments started by recording baseline behavior for 30 min when animals were exposed to COMBO water infused at a rate of 10 μL/min and a temperature of 20.0^{o} C. After this initial 30 min, the heating and cooling stage was adjusted so that the temperature of the perfusate exiting the chamber was 15.0^{o} C. The physiological recordings (from 30min to the end) were taken at this cooler temperature in order

to reproduce the conditions used by Baylor (1942) and Bekker and Krijgsman (1951). These earlier studies maintained animals at 10° C. We found that the round glass cover slip of the chamber-viewing insert would condense water vapor from the room air (~21° C) when the perfusate was maintained at this lower temperature, and this would obscure the view of the animal. Condensation was not a problem when the perfusate was maintained at 15° C. During experiments the perfusate temperature did not deviate more than 0.1° C from the desired temperature (15° or 20° C)

COMBO media was perfused through the chamber for an hour at 15.0° C with video recordings every 10 min. After 60 min the pump rate was increased to 250 μL/min to deliver a test solution through the chamber. The pump rate was then returned back to 10.0 μL/min for the duration of the 60 min exposure period. This method enabled relatively rapid changes of the solution in the chamber, and could be accomplished in less than 10 minutes between recordings. Test solutions containing physostigmine were monitored for 1 hr. In the experiments where animals were pretreated with atropine before the combination of physostigmine plus atropine, atropine alone was perfused for 30 min, and this was then followed by the atropine-physostigmine combination for 1 hr.

In addition to the quantitative measurement of HR and ABR responses using optical tracking, qualitative evaluation of gross changes in behavior were made for the movement of the swimming antennae and the fast rotary tremor of the eye. Motor responses were nominally classified as not suppressed (i.e., showing rhythmic activity) or completely suppressed (no rhythmic activity, appeared to be paralyzed).

Experimental Design and Statistics

A repeated measures ANOVA design with time (min) as the repeated measure was used to analyze the motor responses. The two dependent variables were heart rate (HR) and appendage beat rate (ABR). The independent variables used in the various analyses were parameter (HR/ABR), concentration, and species (*D.pulex/D.magna*). The data illustrated in Figures 3-10 include a baseline pre-drug period of 30 min (-30 to 0 min) and a post-drug period of 60 min (10-60 min), with the time of drug administration starting at the time = 0 min. Contrast analysis was used to compare three means representing baseline at -20, -10 and 0 min to post-drug means at 40, 50 and 60 min for each parameter (HR or ABR). Contrast analysis was also used to compare means after ANOVA. Nominal data depicted in Table 1 was analyzed using log-linear analysis. The categorical variables for log-linear analysis were: species (*pulex, magna*), atropine (Y/N), response (suppressed/not suppressed), and movement type (eye/antennae). The data in figures is expressed as mean +/- standard error, and inferential tests were deemed significant when $P<0.05$.

Drugs and Solutions

Physostigmine (eserine hemisulfate salt), atropine monohydrate sulfate salt, and neostigmine methyl sulfate were obtained from Sigma Aldrich (St. Louis, MO). All drugs were water soluble and were dissolved in COMBO freshwater media for use in experiments (Kilham et al., 1998).

Results

Lowering the water temperature caused a significant decrease (Parameter x Time x Species effect, $P<0.001$) in HR and ABR, both in *D. pulex* (contrast Analysis: 20-30 min versus 80-90 min, $P<0.001$ for both HR and ABR) and in *D. magna* (contrast analysis: 20-30 min versus 80-90 min, $P<0.001$ for both HR and ABR; Figure 2). In addition, a significant Parameter x Species interaction ($P<0.001$) indicated that HR (contrast analysis, $P< 0.001$) and ABR (contrast analysis, $P<0.001$) were significantly higher in *D. pulex* relative to *D. magna*. In *D. magna* ABR was significantly higher than HR before (contrast analysis, $P<0.05$) and after cooling (contrast analysis, $P<0.005$). However, in *D. pulex* HR was significantly higher than ABR before cooling (contrast analysis, $P<0.001$), but not after cooling (contrast analysis, $P>0.50$).

Continuous perfusion of the chamber with the COMBO solution at a rate of 10 μL/min at 15°C, animals could be kept alive in the chamber for more than 5 h (n=6). Perfusion with COMBO solution at 15°C provided stable animal HR and ABR over the duration of the experimental period (Figure 3). The use of a hydraulic valve to switch between two different perfusion syringes on the syringe pump (t=0) that contained the same COMBO solution did not cause a measureable change in HR or ABR (Figure 2; Time effect, Time x Species effect, and Parameter x Species x Time effect were all $P > 0.40$). There was a significant difference in HR and ABR between *Daphnid* species that did not depend on the parameter measured, with basal levels lower in *magna* relative to *pulex* (Species effect, $P<0.001$; Parameter x Species effect, $P>0.10$).

The HR and ABR response of *D. pulex* to three different concentrations of physostigmine is shown in Figure 4. Physostigmine elicited significant concentration-, time-, and parameter-dependent changes in *D. pulex* (Concentration x Time x Parameter effect, $P<0.001$). The 0.5 μM concentration did not elicit any significant effects (contrast analysis, $P>0.40$ for both parameters), but the 1 μM and 2 μM concentrations completely suppressed ABR after 60 min of exposure (contrast analysis, $P<0.001$ in both cases). There were small, but non-significant decreases in HR for the 1 μM and 2 μM concentrations (contrast analysis, $P>0.10$ in both cases). It should be noted that the reduction in ABR observed was most often irregular and the animals often exhibited intermittent pauses in the movement of the appendages followed by resumption of activity at a lower level until activity ceased. Additionally, an increase in post-abdominal contractions resembling the post-abdominal rejection movements described by Kirk (1991) was often observed in response to the perfusion of 1 μM and 2 μM physostigmine. A partial recovery from the effects of exposure to high concentrations of physostigmine was observed following perfusion with drug-free COMBO solution. Recovery was monitored for *D. pulex* and on average it took approximately 60 min for ABR to reach 50% of its ABR reading prior to drug application (n=10). *Daphnia magna* however, took on average approximately 30 min (n=5) to reach 50% of ABR prior to drug application.

Physostigmine also produced concentration-, time-, and parameter-dependent changes in *D. magna* (Concentration x Time x Parameter effect, $P<0.001$). A significant reduction in ABR occurred at the two highest concentrations of physostigmine, 2 and 4 μM (contrast analysis, $P<0.05$; Figure 5). The effect of

physostigmine on HR was not found to be significant (contrast analysis, $P>0.05$ in all cases). When the overlapping concentrations, 1 and 2 μM, were compared, the ABR response of *D. magna* was found to be significantly less sensitive to physostigmine than *D. pulex* (Parameter x Species effect, $P<0.001$; Time x Species effect, $P<0.001$, and Parameter x Time x Species effect, $P<0.001$). An increase in post-abdominal contractions resembling post-abdominal rejection was also observed in response to physostigmine.

To test the hypothesis that the effect of physostigmine on *D. pulex* is mediated by cholinergic receptors pharmacologically similar to vertebrates, the non-selective muscarinic receptor antagonist, atropine, was administered alone or in combination with physostigmine. The effects of atropine on *D. pulex* and *D. magna* HR and ABR can be seen in Figure 6 A and B, respectively. The interaction between parameter, time, and species was not significant (Parameter x Time x Species effect, $P>0.15$). A small, but significant drop in ABR was detected in *D. magna* (contrast analysis, $P<0.01$), but there were no other significant effects of atropine detected on *D. magna* or *D. pulex* (contrast analysis, $P>0.30$ in all cases).

When 10 μM atropine was administered 30 minutes prior to a 2 μM physostigmine challenge in *D. pulex* (Figure 7B), there was a significant attenuation of the effect of physostigmine (Parameter x Treatment effect, $P<0.05$; Parameter x Time x Treatment effect, $P<0.001$) relative to animals that did not receive atropine (Figure 7A). Contrast analysis indicated that there was a significant difference between the atropine plus physostigmine group versus physostigmine alone group at 40, 50, and 60 min for both ABR and HR ($P<0.001$ in both cases). However, the

only significant change in a parameter relative to baseline was found with ABR in the physostigmine alone group (contrast analysis, physostigmine group, ABR: $P<0.001$; HR: $P>0.50$). The HR and ABR were not significantly different from their respective baseline values in the atropine plus physostigmine group (contrast analysis, atropine plus physostigmine group, ABR: $P>0.05$; HR: $P>0.50$).

In *D. magna,* 10 μM atropine pretreatment significantly attenuated the effect of 4 μM physostigmine (Parameter x Treatment, $P<0.005$; Parameter x Time x Treatment, $P<0.05$; Figure 8B) relative to animals that did not receive atropine (Figure 8A). Contrast analysis indicated that there was a significant difference between the group that received physostigmine alone versus atropine plus physostigmine group at 40, 50, and 60 min for ABR ($P<0.01$), but not for HR ($P>0.50$; Figure 8). Additionally, HR did not change significantly relative to initial conditions (i.e., baseline) in either the physostigmine group or the atropine plus physostigmine group (contrast analysis, $P>0.10$ in both cases). Appendage beat rate (ABR) was significantly reduced in the physostigmine group (contrast analysis, $P< 0.001$), but not in the atropine plus physostigmine group (contrast analysis, $P>0.05$). It should be noted that the 10 μM atropine pretreatment appeared to produce a delay in the effect of physostigmine on ABR in both *D. magna* and *D. pulex* rather than producing a complete blockade of the inhibitory response, since there was a trend for a decrease in ABR beginning at 60 min in both species.

Motor function other than HR and ABR was also affected by physostigmine exposure. Rotary tremor of the eye and the swimming antennae were clearly affected by exposure to physostigmine. These other motor functions were not quantified

optically through the software algorithm, but were categorized in a nominal fashion through visual observation. When physostigmine suppression of ABR was at its maximum, fast tremors of the eye and all the other reported eye movements (Frost, 1975) were often completely absent in both species (Table 1). In addition, physostigmine exposure would often cause swimming antennae to cease their beating motion, causing the antennae to lie flat against the carapace. While lying against the carapace, swimming antennae were often observed to show a low amplitude tremor. As can be seen in Table 1, the combination of atropine plus physostigmine reduced the frequency of observing such suppressive effects of the AChE-I on these motor functions.

Neostigmine elicited a significant concentration- and parameter-dependent change in rate (Concentration x Parameter effect, $P<0.05$; Concentration x Time x Parameter effect, $P<0.10$; Figure 9). There was a significant reduction in both HR and ABR activity at the high 20 μM concentration (contrast analysis, $P<0.05$ in both cases), but no significant change for either HR or ABR at the lower, 2 μM concentration of neostigmine (contrast analysis, $P>0.50$ in both cases). Note that the 20 μM concentration of neostigmine did not cause complete inhibition of ABR (Figure 8B) as seen for *D. pulex* exposed to 1 μM physostigmine (see Figure 3B).

Neostigmine elicited a significant concentration- and parameter-dependent change (Concentration x Parameter effect, $P<0.01$; Concentration x Time x Parameter effect, $P<0.001$; Figure 10) in *D. magna*. There was a significant reduction in ABR and significant increase in HR activity at the high 20 μM concentration (contrast analysis,

P<0.01 in both cases), but no significant change for either HR (P>0.20) or ABR (P>0.10) at the lower concentration of neostigmine, 4 μM.

A summary of the effects of physostigmine alone, or in combination with atropine, on eye or swimming antennae movement is shown in Table 1. The observations are expressed as percent of animals that showed complete suppression of the rotatory tremor of the eye or the full sweep of swimming antennae by physostigmine. A complete suppression of motor activity associated with both the eye and antennae movement was often seen in response to physostigmine exposure. This suppressive effect of physostigmine was more clearly concentration-dependent for *D. magna* than for *D. pulex* within the range of concentrations studied. The highest concentration of physostigmine used in the studies of *D. pulex* (2 μM) and *D. magna* (4 μM) was combined with atropine (10 μM) as described above. A log-linear analysis of the frequency data was conducted that included atropine plus physostigmine and the corresponding concentration of physostigmine alone for both species. In a test of all marginal and partial association models, both the partial and the marginal association between the atropine factor and the response factor were significant (P<0.001 in both cases). All the other effects were not found to be significant. This shows that the presence of atropine was associated with a reduced incidence of movement suppression.

Discussion

We studied cholinergic modulation of motor systems in *D. pulex* and *D. magna* using physostigmine as a probe for the presence of endogenous ACh synthetic

activity, and atropine to see if physostigmine-induced responses could be attenuated by a muscarinic receptor antagonist. Since atropine was able to attenuate the effect of physostigmine on ABR, and the effects of physostigmine on the swimming antennae and the eye, a muscarinic-like ACh receptor is implicated in these responses.

Our results are dissimilar to the early pharmacological studies focused on *D. magna* reported by Baylor (1942). We established clear concentration-dependent effects of physostigmine on ABR, but not HR at low concentrations, and were able to see at least partial recovery from these effects over a long washout period. The paralysis of the thoracic appendages, swimming antennae, and eye by physostigmine would undoubtedly eventually be lethal. However, the heart continued to beat during our experimental observations and was not significantly affected during the one-hour exposure to the low physostigmine concentrations. Baylor (1942) reported inhibition of HR by physostigmine, but also found his results difficult to interpret and described physostigmine as toxic. Given the paralysis of other motor systems at 1 to 4 μM, we did not use physostigmine at concentrations higher than 4 μM, and therefore inhibition of HR might be seen at higher concentrations. Since Baylor did not combine atropine with physostigmine, it is not clear if the reduction in HR was due to an increase in ACh. Baylor did observe that fairly high concentrations of ACh could cause moderate reductions in HR, suggestive of the presence of ACh receptors that could modulate HR. However, atropine was not used to determine if this response was receptor mediated. Our accompanying paper (Hannan et al., submitted) examines the effects of direct receptor agonists and antagonists on HR

and ABR. Baylor (1942) found that high concentrations of atropine caused a small decrease in HR. We found that 10 μM atropine antagonized the effects of physostigmine on ABR, but this concentration of atropine alone did not lower HR.

Bekker and Krijgsman (1951) studied both *D. pulex* and *D. magna* and reported there were no species-dependent differences in the responses of the *Daphnid* hearts in their study. In the present study we see clear species-dependent differences in the sensitivity of ABR to physostigmine-induced inhibition, motor function Bekker and Krijgsman did not apparently examine. Bekker and Krijgsman found that relatively high concentrations of ACh, the muscarinic agonist, pilocarpine, and the AChE-I, tetraethyl pyrophosphate, slowed HRs, while atropine increased HRs. We did not observe significant physostigmine-induced decreases in HR from physostigmine in the concentration range examined. The concentration of atropine we employed in the present study did not significantly alter HR. Additional studies with direct receptor antagonists are described in Chapter 2.

As in studies by Baylor (1942) and Bekker and Krijgsman (1951), mostly at 10^{o} C, our study utilized cool temperatures (15^{o} C) to evaluate the physiology of *Daphnia*. Although the temperature difference seems to be an unlikely reason for dissimilar findings, it cannot be entirely ruled out. However, more likely reasons for differences between our findings and those of the earlier two studies include: animal health (e.g., potential effect of cotton fibers on movement of appendages), flow rate, temperature stability, selection of drug concentrations, methods for quantification (e.g., high quality digital videos with computer assisted analysis), and the clonal differences.

Our results clearly demonstrate effects of an AChE-I on motor function in two *Daphnid* species that vary according to particular target organ considered, thoracic appendages (i.e., ABR), heart (i.e., HR), swimming antennae, or the eye. Among these four targets, the heart was the least affected by exposure to low micro-molar concentrations of physostigmine. The significant concentration-dependent inhibition of ABR represents a very steep concentration-response curve that could almost be characterized as all or nothing, where total inhibition of ABR is achieved by just doubling an ineffective threshold concentration. This steep concentration-response relationship is most likely due to the fact that the principal target of physostigmine is an enzyme that degrades ACh rather than an ACh receptor. Once a relatively specific threshold of enzyme activity has been crossed, critical levels of ACh accumulate, and normal cholinergic signaling may be impaired due to excessive receptor stimulation. The insecticide, diazinon, an organophosphate AChE-I, was found to immobilize *D. magna* once AChE reached a threshold of activity that was 40% of control (Kretschmann et al., 2011). It is very likely that the responses to physostigmine observed in this study also involve reaching a critical threshold of AChE activity. Once enzyme activity decreases further, the high levels of ACh overwhelm the capacity to shut down the chemical signal.

The physostigmine and atropine results are consistent with ACh playing a role in the regulation of the beating of thoracic appendages, the beating of the swimming antennae, and movement of the eye. These pharmacological results provide support for the importance of ACh as a chemical messenger in *Daphnia*. The AChE-I, neostigmine has a quaternary nitrogen group, and is therefore more polar than

physostigmine, which has only tertiary nitrogens. The finding that a high concentration of neostigmine was less effective than much lower concentrations of physostigmine in inhibiting ABR suggests that there is a significant diffusion barrier between the more polar neostigmine AChE-I and the target enzyme, AChE. This study does not address the location of the target enzyme, AChE-I. To the best of our knowledge, the anatomical location of acetylcholine, the synthetic enzyme, choline-acetyltransferase, or AChE has not been mapped in *Daphnia.*

Acetylcholine may be one of the oldest transmitters, and it is used for intercellular communication between neuronal and non-neuronal tissue (Kawashima et al., 2007; Thany and Tricoire-Leignel, 2011; Yamada et al., 2005). Acetylcholine has been identified as a neurotransmitter in arthropods (Sattelle and Breert, 1990; Walker et al., 1996; Walker and Holden-Dye, 1989) and is found in high concentration in the CNS (Sattelle and Breert, 1990). A recent study by McCoole et al. (2012) demonstrated the presence of choline-acetyltransferase, vesicular ACh transporter, acetylcholinesterase, nicotinic receptor subunits, and the muscarinic receptor within the *Daphnid* genome. The presence of cholinergic neurons has also been clearly demonstrated in many arthropods (Sattelle and Breert, 1990; Walker et al., 1996; Walker and Holden-Dye, 1989), including crustaceans (Logsdon et al., 2006; Yazawa et al., 1998). The finding that critical elements of cholinergic systems can be found in the genome, and the well-documented sensitivity of *Daphnia* to the lethal effects of insecticides that target AChE (e.g., organophosphates and carbamates) strongly suggest that ACh plays a critical physiological role as a chemical messenger in these animals.

What is apparently not yet known is the anatomical location of the source of ACh that could be eliciting responses to physostigmine in target organs. Stein et al. (1966) conducted EM studies of the *D. magna* heart, but did not describe any nervous innervation of the heart. This leads to the suggestion that the pacemaker activity of the *Daphnid* heart maybe myogenic (Bekker and Krijgsman, 1951; Stein et al., 1966). The relatively small and statistically insignificant effects of the one-hour physostigmine application on heart rate in the present study seem consistent with this observation. However, strong and statistically significant responses of the thoracic appendages, swimming antennae, and eye to physostigmine were observed, and this might suggest control of motor function by peripheral cholinergic nerves. However, the neuromuscular junction of insects, such as *Drosophila melanogaster* has been shown to utilize glutamate as a neurotransmitter (Keshishian et al., 1996). If this proved to be a universal theme among all arthropods, then the motor activity influenced by cholinergic stimulation in *Daphnids* may not be at the level of the peripheral neuromuscular junction, but in the CNS. To add to the complexity of cholinergic function in *Daphnids,* the findings reported in Chapter 2 describe species-dependent responses in *D. pulex and D. magna* elicited by direct receptor agonists of cholinergic receptors.

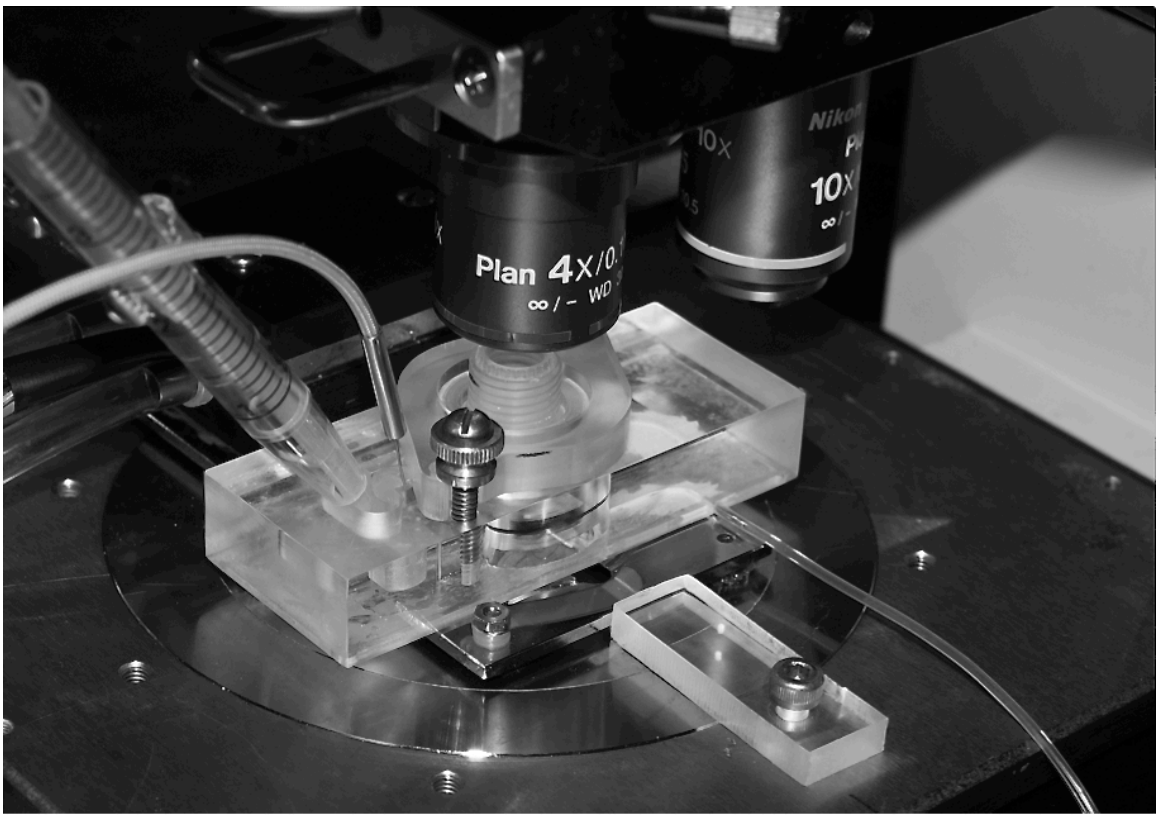

Figure 1 – Aquatic Chamber

Aquatic chamber with setup; microscope, temperature probe, inlet and outlet tubes.

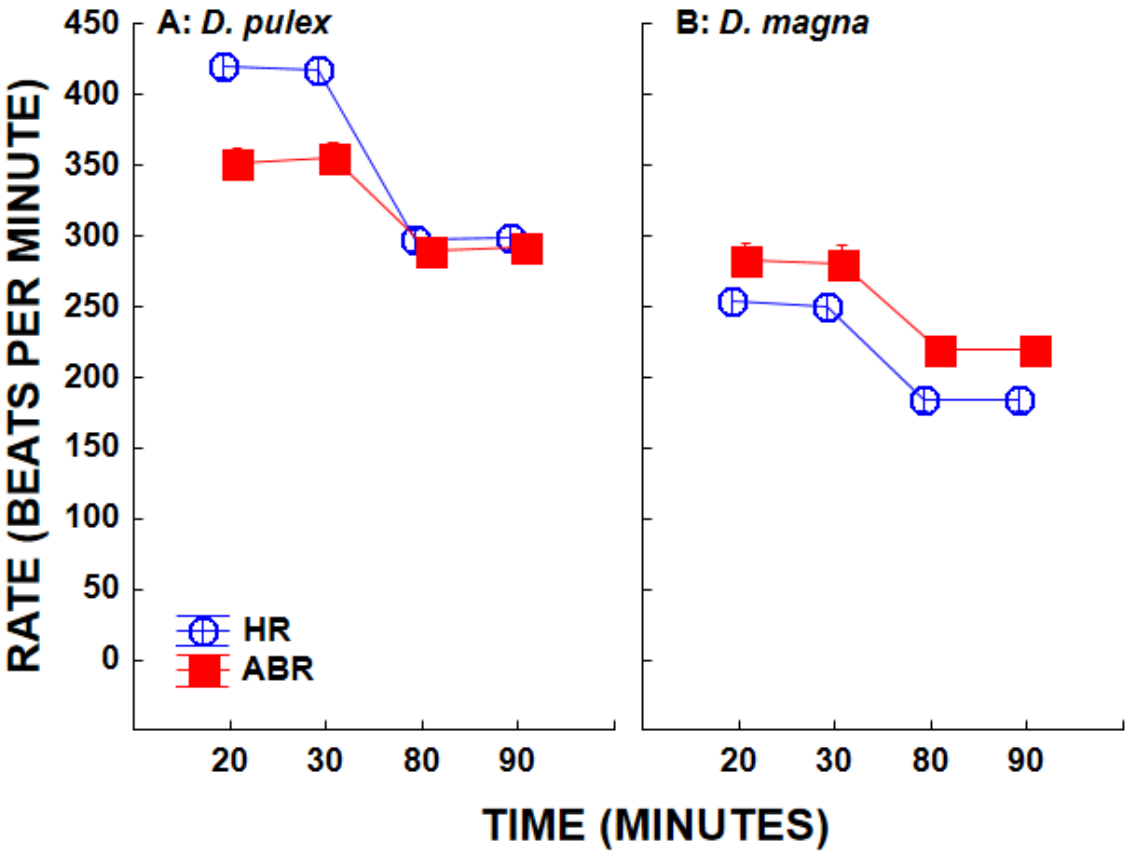

Figure 2 – Effect of temperature on *Daphnia pulex* and *Daphnia. magna*

Time points were chosen to showcase the effect of temperature on *D. pulex* (left) and *D. magna* (right) HR and ABR. Temperature was lowered from 20°C at 30 min to 15°C for the remainder of the experiment.

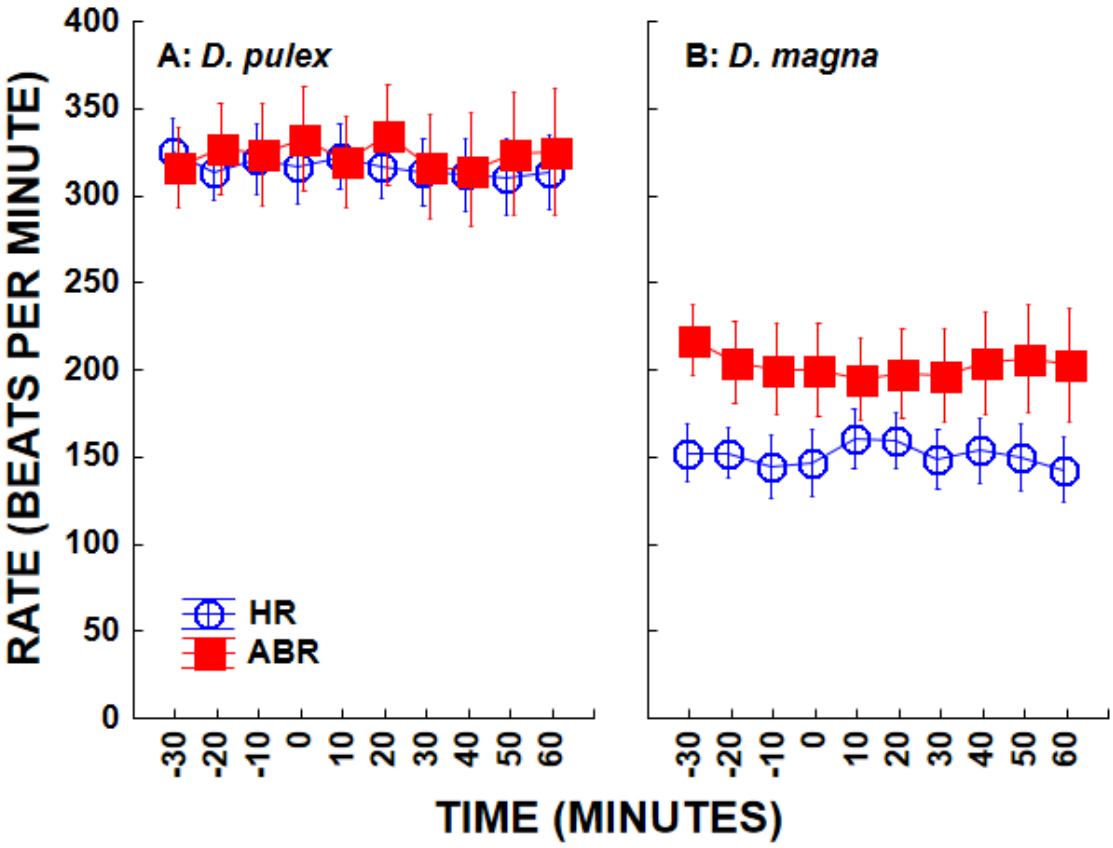

Figure 3 – Exposure of Combo to *Daphnia pulex* and *Daphnia magna*

D. pulex (A) and *D. magna* (B) were exposed to COMBO solution alone, with no drug solution present. The hydraulic valve was switched between two identical COMBO solutions at time=0.

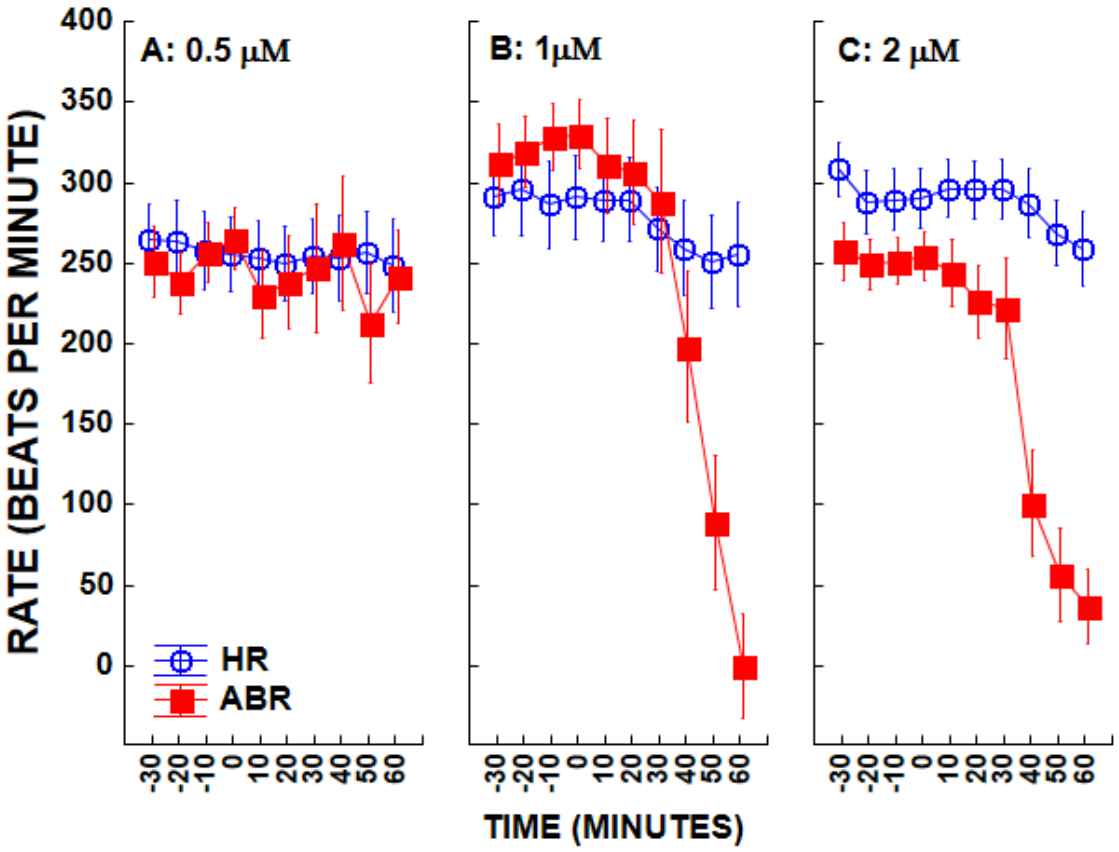

Figure 4 – Effects of Physostigmine on *Daphnia pulex*

Effect of physostigmine; concentrations 0.5µM (n=5), 1 µM (n=4), and 2 µM (n=8) on the HR and ABR of *D. pulex*. Error bars represent ± one standard error.

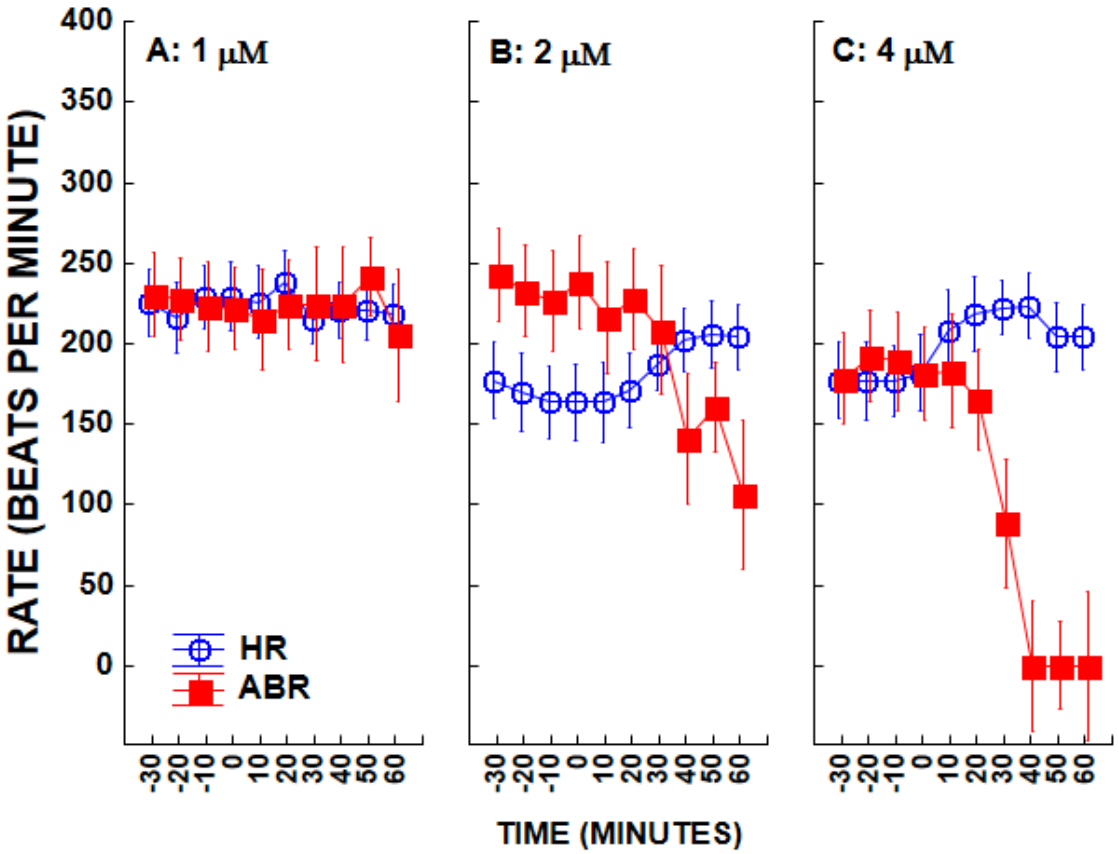

Figure 5 – Effects of Physostigmine on *Daphnia magna*

Effect of physostigmine; concentrations 1 μM (n=5), 2 μM (n=4), and 4 μM (n=4) on the HR and ABR of *D. magna*. Error bars represent ± one standard error.

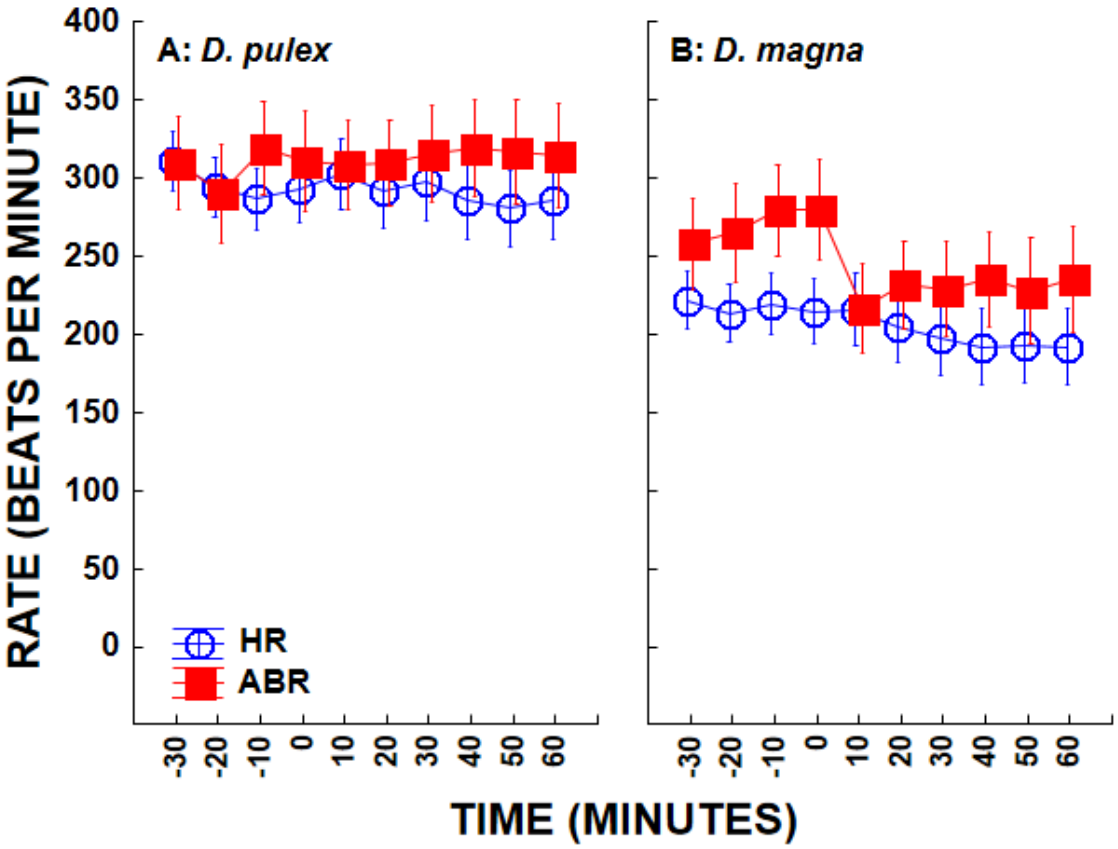

Figure 6 – Effects of Atropine on *Daphnia pulex* and *Daphnia magna*

Effects of atropine (10mM) on HR and ABR of both *D. pulex* (A) and *D. magna* (B). Error bars represent ± one standard error.

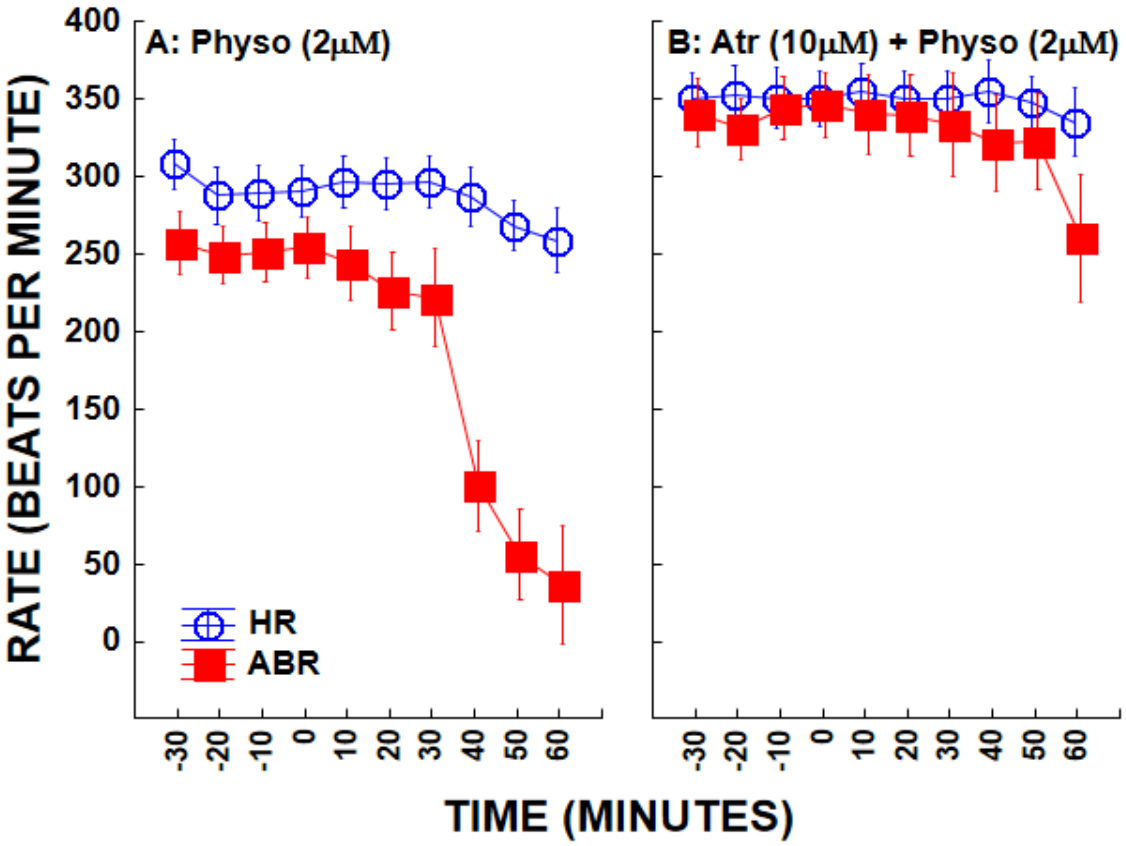

Figure 7 – Effects of Atropine pretreatment and Physostigmine on *Daphnia pulex*

Panel A shows the effect physostigmine (2 μM) (n=8) has on HR and ABR of *D. pulex*. Panel B showcases the effect an atropine pretreatment (10 μM) has on HR and ABR when followed by an atropine (10 μM) and physostigmine (2 μM) combination (n=7). The dotted line (-20 min) in B marks introduction of atropine (10 μM) alone. The complete line (0 min) in both marks the introduction of physostigmine, (A) and atropine – physostigmine (B) solutions. The data depicted in Panel A is from figure 4C. Error bars represent ± one standard error.

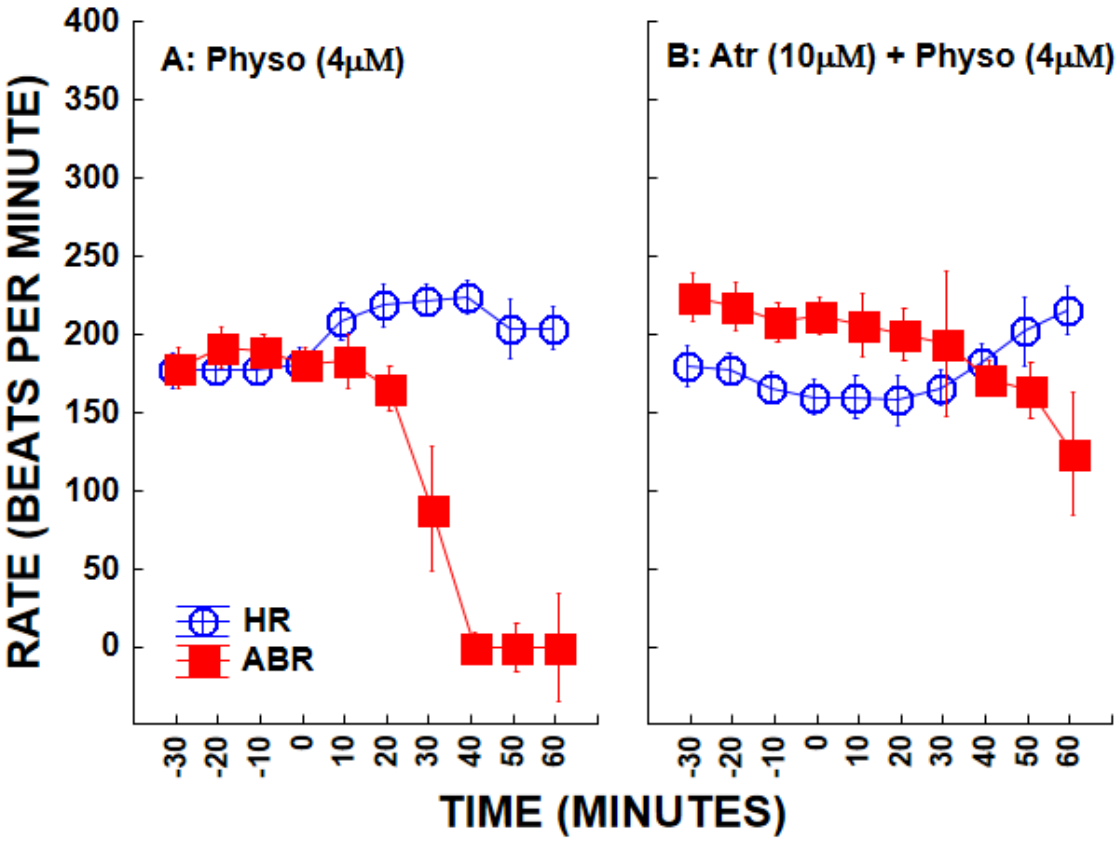

Figure 8 – Effects of Atropine pretreatment and Physostigmine on *Daphnia magna*

Panel A shows the effect physostigmine (4 μM) has on HR and ABR of *D.* magna (n=4). Panel B, in comparison, showcases the effect an atropine pretreatment (10 μM) has on HR and ABR when followed by an atropine (10 μM) and physostigmine (4 μM) combination (n=3). The -20 min time point in B marks introduction of atropine (10 μM) alone. The 0 min time point in both marks the introduction of physostigmine, (A) and atropine – physostigmine (B) solutions. The data depicted in Panel A is from Figure 5C. Error bars represent ± one standard error.

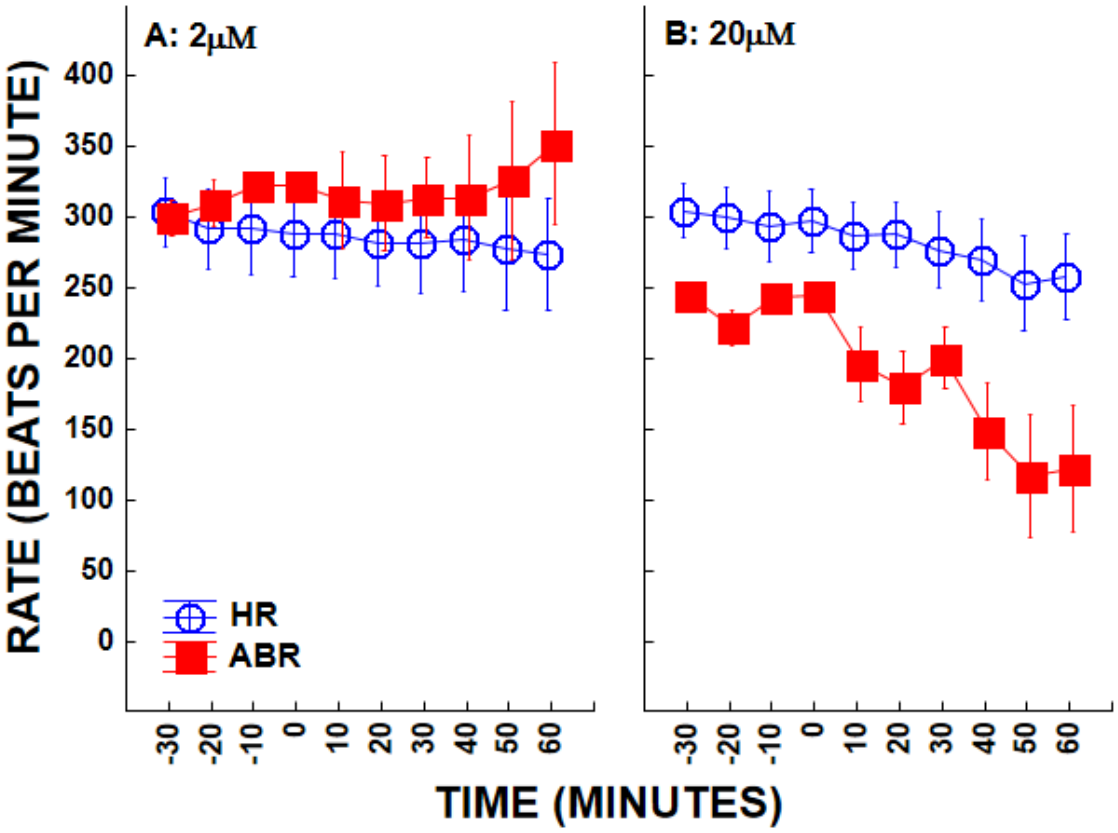

Figure 9 – Effects of Neostigmine on *Daphnia pulex*

Effect of the acetycholinesterase inhibitor neostigmine on HR and ABR of *D. pulex* at concentrations 2 µM (A) and 20 µM (B). Error bars represent ± one standard error.

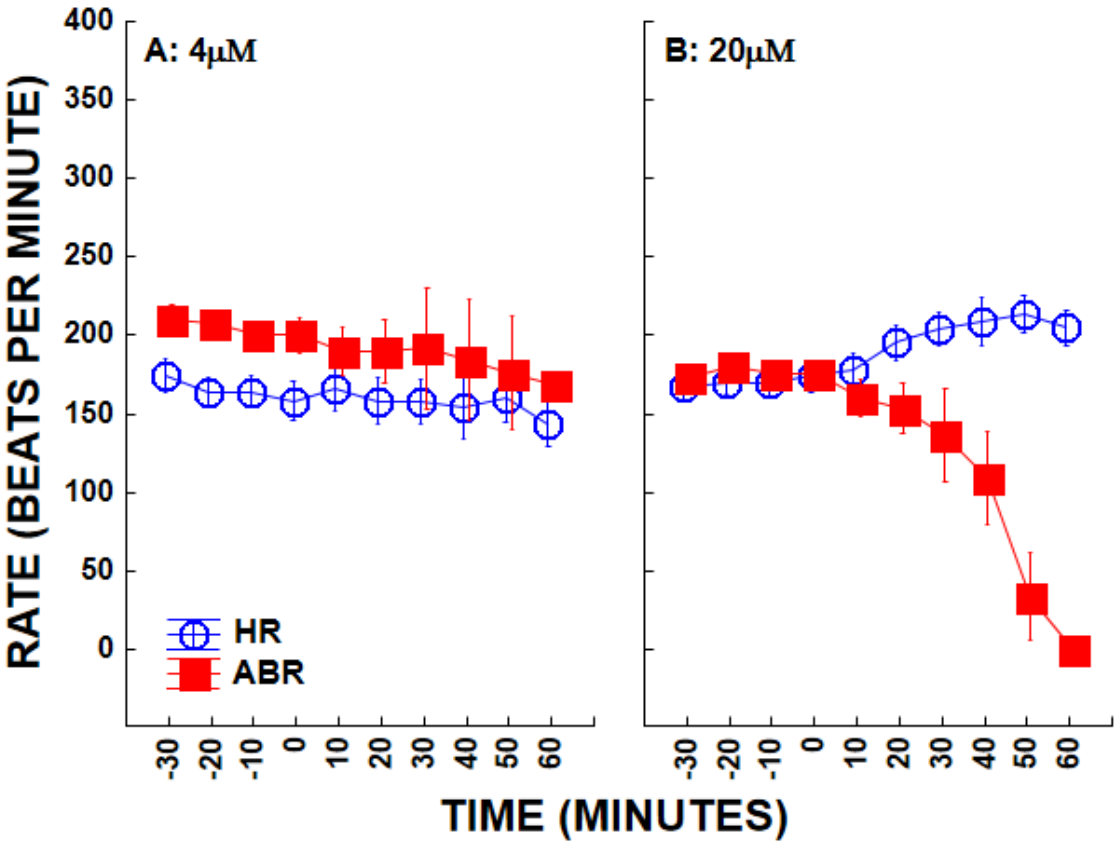

Figure 10 – Effects of Neostigmine on *Daphnia magna*

Effect of the acetycholinesterase inhibitor neostigmine on HR and ABR of *D. magna* at concentrations 4 µM (A) and 20 µM (B). Error bars represent ± one standard error.

Table 1

Motor activity: Paralysis of fast rotary tremor of the eye and the rhythmic activity of the swimming antennae (S.A.) by physostigmine.

Drugs (μM)	Eye - *D. pulex* % (n)	Eye - *D. magna* % (n)	S.A. - *D. pulex* % (n)	S.A. - *D. magna* % (n)
Physo (0.5)	60 (5)		60 (5)	
Physo (1.0)	25 (4)	20 (5)	50 (4)	20 (5)
Physo (2.0)	62 (8)	50 (4)	50 (8)	50 (4)
Physo (4.0)		100 (4)		100 (4)
Physo (2.0) + Atr (10)	14 (7)		14 (7)	
Physo (4.0) + Atr (10)		0 (3)		33 (3)

Chapter 2: Comparison of *Daphnia pulex* and *magna* responses to cholinergic stimulation: Direct receptor agonists.

Introduction

Although there have been few pharmacological studies in *Daphnia*, the nature of cholinergic receptor mediated responses is of considerable interest. *Daphnia* are a keystone species with an extensive literature on their ecology, and they are extensively used in water quality testing, and in the evaluation of the toxicity of water contaminants such as pesticides. *Daphnia* are arthropods known to be very sensitive to many pesticides, especially the insecticides that affect cholinergic function (Jansen et al., 2011a; Jansen et al., 2011b; Kretschmann et al., 2012; Manar et al., 2012; Pavlaki et al., 2011; Zein et al., 2014; Zhang et al., 2012).

Since the *D. pulex* genome has been sequenced (wfleabase.org) and *Daphnia* are considered a model organism by NIH and are particularly well adapted to respond to biotic and abiotic stressors (Colbourne et al., 2011), there is increased interest in the use of *Daphnia* for toxicogenomic studies (Shaw et al., 2008). Because *Daphnia* are parthenogenetic, produce both asexually and sexually, reproduce rapidly, and are able to produce diapausing eggs, they are also particularly well suited for evolutionary studies (Cristescu et al., 2012; deWaard et al., 2006; Omilian et al., 2006; Walsh et al., 2012; Walsh and Post, 2012), as well as studies evaluating the effects of multiple stressors, including chemical stressors, on adaptation and speciation (Altshuler et al., 2011). The recent identification of choline-acetyltransferase, vesicular acetylcholine transporter, acetylcholinesterase, nicotinic receptor subunits, and a muscarinic receptor in the *Daphnid* genome (McCoole et al.,

2012) leads to critical questions concerning the regulatory role of the cholinergic system in *Daphnia,* its potential role in adaptation, and potential contributions to the field of functional genomics (Eads et al., 2008; Nikinmaa and Rytkonen, 2011).

Early pharmacological studies of *Daphnia* have characterized the responses of the heart, appendages, and gut to various pharmacological agents (Baylor, 1942; Bekker and Krijgsman, 1951; Obreshkove, 1941; Oconnor, 1950; Sollmann and Webb, 1941). In Chapter 1, we examined the regulation of the heart and appendages by acetylcholine (ACh) using the acetylcholinesterase inhibitor (AChE-I), physostigmine, to increase ACh levels. A computer-based optical analysis of movement revealed that ACh likely modulates the activity of the thoracic appendages, the activity of the swimming antennae and the fast rotary tremor of the eye. The thoracic appendages, swimming antennae and eye were found to be particularly sensitive to the suppression of motor activity elicited by physostigmine. Although there was a trend toward changes in heart rate (HR) elicited by physostigmine exposure, significant alterations in HR were not detected in the concentration range and sample sizes studied.

Baylor (1942) and Bekker and Krijgsman (1951) have previously reported that ACh, in the low micro-molar concentration range, decreases *Daphnid* HR. Bekker and Krijgsman reported that the muscarinic agonist, pilocarpine (50 μM), also decreased HR. Baylor found that the muscarinic antagonist, atropine, caused small decreases in HR (~ 0.1 to 10 μM), and concluded that this was most likely a toxic effect of atropine. However, Bekker and Krijgsman found that 60 to 250 μM concentrations of atropine increased HR. Neither study examined the interaction

between atropine and direct receptor agonists or AChE-Is. Both the Baylor study and the Bekker and Krijgsman study utilized *D. magna* as the experimental subject. Bekker and Krigjsman also included *D. pulex* in their investigation, but did not differentiate the *D. magna* or *D. pulex* responses – stating that "No differences were observed in the reactions of the hearts of these two species." In Chapter 1 we found that *D. pulex* was significantly more sensitive to physostigmine than *D. magna.*

The goal of the present study is to revisit the pharmacological study of the *Daphnid* cholinergic system using an aquatic chamber with a highly regulated media flow rate and temperature, and computer-aided quantification of movement. Chapter 1 provided strong evidence of cholinergic modulation of the thoracic appendages, swimming antennae and fast rotary tremor of the eye, and significant species-dependent differences in sensitivity to the AChE-I, physostigmine. This study focuses on direct receptor agonists and further examines the hypothesis that there are species-dependent differences in cholinergic regulation of cardiac and motor activity.

Methods

Animals

Daphnia pulex and *D. magna* housed in 4 L jars were kept in an incubator at a temperature of 21°C, and at a light/dark cycle of 16/8 h using broad-spectrum fluorescence lights. The media consisted of an artificial freshwater solution, COMBO (Kilham et al., 1998). Animals were fed a 50/50 mixture of *Ankistrodesmus falcatus* and *Chlamydomonas reinhardii* algae 3 times per week.

Aquatic chamber

An aquatic chamber was used with bright field microscopy to observe and record animal movement. The description of the chamber can be found in the Chapter 1. The setup holds the animal at a fixed location inside the chamber and allows for the free movement of appendages and antennae. Media solutions containing various drugs were delivered at flow rates of 10 μL/min (i.e., continuous measurement of responses) or 250 μL/min (i.e., for changing solutions in aquatic chamber).

Animal Preparation

Adult female *D. magna* and *D. pulex* were selected for the experiments based on size. As described previously (Hannan et al. submitted), animals were glued to the tip of a 33-guage-syringe needle with a very small drop of cyanoacrylate glue (< 150 nl). The needle holding the animal was placed into a holder within the aquatic chamber, filled with COMBO media, and the top chamber-viewing insert fixed into place. All animals were able to freely move their swimming antennae and appendages while in the chamber.

Drug perfusion experiments

A detailed description of the experimental setup can be found in Chapter 1. Consistent with this previous chapter, experimental recording periods started with a 30 min baseline of COMBO water infused at a rate of 10 μl/min and a temperature of 20.0°C. After the 30-minute baseline period, the Peltier pump was adjusted so that the temperature of the perfusate exiting the chamber was 15.0°C. Physiological

recordings were taken at this cooler temperature in order to reproduce the cooler conditions used by Baylor (1942) and Bekker and Krijgsman (1951). The COMBO media was then perfused through the chamber for an hour at 15.0°C with video recordings every 10 min. At 60 minutes the pump rate was increased to 250 μL/min to deliver a test-solution over a period of approximately 7 minutes, and then the pump rate was returned back to 10.0 μL/min for the duration of the 60 min test-solution exposure period (nicotine, oxotremorine, pilocarpine). In the experiments where animals were pretreated with atropine before the combination of nicotine or oxotremorine plus atropine, atropine alone was perfused for 30 min prior to the atropine-nicotine/oxotremorine exposure combination for 1 h.

Experimental Design and Statistics

A repeated measures ANOVA design with time (min) as the repeated measure was used to analyze motor responses. Two dependent variables, heart rate (HR) and appendage beat rate (ABR), were quantified every 10 min using the 2D-Tracking module from Image-Pro Plus 7.0. The independent variables used for statistical analysis were parameter (HR/ABR), concentration, and species (*D. pulex/D. magna*). Contrast analysis was used to compare three means representing baseline at -20, -10 and 0 min to post-drug means at 40, 50 and 60 min for each parameter (HR or ABR). Contrast analysis was also used to make specific comparisons across species and parameters. All line graphs for drug studies include a baseline pre-drug period of 30 min (-30 to 0 min) and a post-drug period of 60 min (0-60 min), with the time of drug administration starting at time = 0 min. Data is expressed as mean ± one standard error, and inferential tests were deemed significant when $P<0.05$.

Drugs and Solutions

Nicotine hydrogen tartrate salt, oxotremorine sesquifumarate salt, atropine monohydrate sulfate salt and pilocarpine hydrochloride were obtained from Sigma Aldrich (St. Louis, MO). All drugs were water soluble and dissolved in COMBO artificial freshwater media (Kilham et al., 1998).

Results

Lowering the water temperature from 20°C (initially held for 30 min) to 15°C caused a significant decrease (Parameter x Time x Species effect, $P<0.001$) in HR and ABR, both in *D. pulex* (contrast Analysis: 20-30 min versus 80-90 min, $P<0.001$ for both HR and ABR; Figure 1) and in *D. magna* (contrast analysis: 20-30 min versus 80-90 min, $P<0.001$ for both HR and ABR). In addition, a significant Parameter x Species interaction ($P<0.001$) indicated that HR (contrast analysis, $P< 0.001$) and ABR (contrast analysis, $P<0.001$) were significantly higher in *D. pulex* relative to *D. magna*. In *D. magna* ABR was significantly higher than HR before (contrast analysis, $P<0.001$) and after cooling (contrast analysis, $P<0.001$). However, in *D. pulex* HR was significantly higher than ABR before cooling (contrast analysis, $P<0.001$), but not after cooling (contrast analysis, $P>0.50$)

Oxotremorine elicited a significant concentration-and time-dependent reduction in ABR rate and a smaller, but significant concentration- and time-dependent reduction in HR (Concentration x Time x Parameter effect, $P<0.001$; Figure 2). Significant reductions in ABR were observed during exposure to 8, 32 and 128 μM oxotremorine, and smaller, but significant reductions in HR (bradycardia)

were observed at 32 μM and 128 μM (Contrast Analysis, $P<0.05$ relative to baseline in all cases).

Oxotremorine elicited a significant concentration- and time-dependent change in both ABR rate and HR (Concentration x Time x Parameter effect, $P<0.001$: Figure 3). Significant reductions in ABR were observed at concentrations of 32 and 128 μM (Contrast Analysis, $P<0.05$ relative to baseline in both cases). In contrast to *D. pulex,* oxotremorine elicited a significant, but opposite effect on HR (tachycardia) at 32 and 128 μM (Contrast Analysis, $P<0.05$ relative to baseline in both cases). A combined analysis of *D. pulex* and *D. magna* indicated that the difference between species was dependent on concentration, time, and parameter (species x Concentration x Time x Parameter effect, $P<0.001$). The oxotremorine-induced reduction in ABR was often found to be irregular for both species, with intermittent pauses in the movement of the appendages followed by resumption of activity at a lower level until all movement stopped.

The muscarinic antagonist, atropine (10 μM), completely blocked the effect of 32 μM oxotremorine on ABR and HR in both species ($P>0.50$, Species X Time X Parameter Effect, Figure 4 and 5, respectively). When pilocarpine, a muscarinic agonist, was administered to *D. Pulex* it elicited a significant concentration- and time-dependent reduction in ABR and HR (Concentration x Time x Parameter effect, $P<0.005$, Figure 6). The pilocarpine concentration of 32 μM did not elicit a significant decrease in HR (Contrast Analysis, $P>0.15$ relative to baseline), but there was a trend towards decreasing ABR (Contrast Analysis, $P\sim0.078$ relative to baseline). The 128 μM concentration of pilocarpine elicited a significant decrease in

both HR (Contrast Analysis, P< 0.01 relative to baseline) and ABR in *D. pulex* (Contrast Analysis, P < 0.005 relative to baseline). When pilocarpine was administered to *D. magna,* it elicited a significant Time x Parameter effect (P<0.001), but not a significant Concentration x Time x Parameter effect (P>0.50), indicating that the two concentrations of pilocarpine elicited similar effects on HR and ABR over time (Figure 7). In *D. magna,* both the 32 μM (Figure 7A) and 128 μM pilocarpine (Figure 7B) elicited significant, but opposite changes in HR (increase) and ABR (decrease) (Contrast Analysis, P<0.01 in all cases). The direction of responses to pilocarpine (increase/decrease) is similar in direction to those elicited by oxotremorine in each species respectively, and a significant parameter- and time-dependent difference between species was found for the pilocarpine responses when both species were included in ANOVA (Species x Parameter x Time effect, P<0.001). The reduction in ABR elicited by both pilocarpine and oxotremorine was also accompanied by an increase in post-abdominal contractions resembling post-abdominal rejection.

Nicotine produced a concentration- and time-dependent reduction of ABR and HR in *D. pulex* (Concentration x Time x Parameter effect, P<0.001; Figure 8). The reduction in ABR was significant across all three concentrations tested, 4, 16 and 64 μM (Contrast Analysis, P<0.05 relative to baseline in all cases). The effect of nicotine on HR was smaller, but a significant reduction in HR (bradycardia) was elicited at 4 and 16 μM (Contrast Analysis, P<0.05 in both cases, relative to baseline) with a small non-significant decrease at 64 μM (Contrast Analysis, P~0.072 relative to baseline).

Nicotine elicited a time- and concentration-dependent effect (Concentration x Time x Parameter effect, $P<0.001$; Figure 9). The 4 μM concentration of nicotine did not exert significant effects on either HR or ABR (Contrast Analysis, $P>0.10$ relative to baseline in both cases). The 16 and 64 μM concentration of nicotine produced significant reductions in ABR in *D. magna* (Contrast Analysis, $P<0.05$ relative to baseline in both cases). Only the highest concentration of nicotine, 64 μM, caused a small trend towards an increase (tachycardia) in HR (Contrast Analysis, $P\sim 0.076$).

Nicotine (16 μM) elicited significant effects in both species in the presence of 10 μM atropine (Concentration x Time x Parameter effect, $P<0.001$, both cases; Figure 10 and 11). Exposure to 16 μM nicotine did not produce significant effects on HR in either species (Contrast Analysis, $P>0.50$ relative to baseline in both cases). Atropine did not block the significant reduction in ABR elicited by nicotine in *D. pulex* (Contrast Analysis, $P<0.001$ relative to baseline) or in *D. magna* (Contrast Analysis, $P<0.05$ relative to baseline).

Discussion

Using the AChE-I, physostigmine, as a pharmacological tool, our previous report provided strong evidence, although indirect, that ACh participates in the regulation of a number of *Daphnid* motor functions, including the beating of thoracic appendages (ABR), the movement of swimming antennae, and the fast rotary tremor of the eye. This study extends the previous findings by examining the *Daphnid* responses to direct ACh receptor agonists and the interaction between these selective receptor agonists and the muscarinic antagonist, atropine. The basal mean

levels of ABR and HR, and the effects of lowering water temperature on these physiological parameters, are extremely close in magnitude to those reported in Chapter 1, and this suggests that the animals used in the current study were in a very similar physiological state.

In contrast to the claim by Bekker and Krijgsman (1951), I have found significant species-dependent differences in both sensitivity and nature of responses to cholinergic stimulation in *D. pulex* and *D. magna*. It should also be noted that at present I do not know the extent of within versus among species variation in responses to cholinergic stimulation, so my results cannot be generalized to all clones of each species, but rather demonstrate significant variation in sensitivity and responsiveness to cholinergic stimulation within the family, *Daphniidae.*

In both species, physostigmine, oxotremorine, pilocarpine and nicotine were found to elicit significant reductions in ABR at relatively low concentrations. The inhibitory effects of nicotine on ABR were not blocked by the muscarinic antagonist, atropine, in either species. These results suggest that stimulation of both nicotinic- and muscarinic-like ACh receptors can inhibit ABR. The ability of the same concentration of atropine to significantly attenuate the effects of physostigmine on ABR suggests that AChE-I induced decreases in ABR may be predominantly mediated through activation of muscarinic receptors.

Similarly, relatively low concentrations of physostigmine were able to suppress the motor activity of both the swimming antennae and the fast rotary tremor of the eye in both species, and our results suggest that these effects can be blocked by atropine (Hannan et al., submitted). Since a full blockade of the

physostigmine-induced suppression of motor activity was not achieved in all of the experiments, a concentration higher than 10 μM may be required to completely block the physostigmine response. Interestingly, with the exception of one *D. magna* receiving the highest concentration of nicotine, a suppression of swimming antennae and eye tremor activity was not observed in either species for the direct acting receptor agonists, oxotremorine or nicotine (Table 1). This may suggest that either higher levels of stimulation of a given receptor subtype is required to elicit a physostigmine-like response or that such suppression of activity requires the simultaneous stimulation of both receptor-subtypes.

A surprising finding in the present study was opposing species-dependent HR responses elicited by muscarinic receptor stimulation. Both oxotremorine and pilocarpine elicited concentration-dependent decreases in HR in *D. pulex*, and concentration-dependent increases in HR in *D. magna*. The ability of atropine to attenuate the response to oxotremorine in both species is consistent with these opposing species-dependent responses being elicited by a muscarinic-like receptor. Although the HR responses elicited by physostigmine were not significant for the concentration-range and sample sizes studied (Hannan et al., submitted), the trend for the change in HR was consistent with the opposite responses of the two species to stimulation with the direct muscarinic-receptor agonists (oxotremorine and pilocarpine).

The regulation of HR in higher crustaceans is considered to be neurogenic, with pacemaking accomplished by cardiac ganglia activity (Cooke, 2002; McMahon et al., 2002; Yazawa et al., 1998). However, the regulation of *Daphnid* HR by the nervous

system has not been demonstrated, and it has been suggested that the pacemaker mechanism is myogenic (Bekker and Krijgsman, 1951; Stein et al., 1966). Bekker and Krijgsman (1951) suggested that ACh regulates the myogenic pacemaker of the *Daphnid* heart through "extrinsic cholinergic nerves impinging on the pacemaker." It seems unlikely that the *Daphnid* heart receives significant cholinergic innervation, because at the concentrations where large effects of physostigmine on ABR, eye, and swimming antennae movement were observed, only small statistically insignificant changes in heart rate occurred. However, it still remains possible that higher physostigmine concentrations could elicit significant effects on HR. If such effects were observed experimentally, it would be critical to ensure that these were not nonspecific or toxic. Additionally, significant and opposite responses in HR were observed for the two species with direct receptor muscarinic agonists. If innervation of the *Daphnid* heart were glutamatergic, as reported in other crustaceans (Delgado et al., 2000; Sakurai and Yamagishi, 2000; Yazawa et al., 1998), then it is possible that a glutamatergic nerve innervating the heart could be regulated centrally by muscarinic receptors. If the *Daphnid* heart is not innervated, then the possibility remains that ACh may be released from non-neuronal tissue that is located in reasonable proximity to the heart. It is also possible that muscarinic receptors associated with the myocardium may be present in adult animals, but endogenous ACh does not normally stimulate them.

To the best of our knowledge, ACh and the components of the cholinergic system have not been anatomically mapped in *Daphnia*, so specific inferences regarding the source of ACh, the anatomical location of ACh receptors, or the target

tissue (muscle or neuron) cannot be made at this time. Since glutamate is known to be associated with the insect neuromuscular junction (Faeder et al., 1970; Keshishian et al., 1996) the neuromuscular junction of higher crustaceans (Logsdon et al., 2006; Yazawa et al., 1998), a likely candidate for the identity of the neurotransmitter associated with the *Daphnid* neuromuscular junction may also be glutamate. However, Stern and Bicker (2008) suggest that neurotransmitter associations with the neuromuscular junction may not be well suited to tracing phylogenetic relationships. The findings, that physostigmine suppresses the motor activity of the thoracic appendages, swimming antennae and eye, that atropine blocks this suppressive effect of physostigmine, and that atropine alone does not have a significant effect on these motor functions (e.g., paralysis), do not support a primary role for ACh at the *Daphnid* neuromuscular junction.

ACh is found in high concentrations in the insect CNS, but not at the neuromuscular junction (Keshishian et al., 1996; Sattelle and Breert, 1990). If the assumption was made that that the central nervous system rather than the neuromuscular junction mediates the major effects of the cholinergic agents, then central pattern generating circuits might be a logical place for such cholinergic modulation of rhythmic behavior to occur (Selverston, 2010). Cholinergic modulation of pattern generators associated with insect flight (Buhl et al., 2008) and respiration (Zafeiridou and Theophilidis, 2004) have been reported in arthropods. Additionally, central pattern generators, such as those associated with the stomatogastric ganglion, have been identified in crustaceans (Dickinson and Nagy,

1983; Nagy and Dickinson, 1983), and are also modulated by the cholinergic mechanisms (Katz and Harris-Warrick, 1989, 1991).

The rhythmic beating of the thoracic appendages generates a "feeding current" that flows through the ventral opening of the carapace. This feeding current has two major functions – one is to provide food particles for ingestion, and the other is to create a continual flow of water for ventilation (Pirow and Buchen, 2004; Pirow et al., 1999). In *D. magna*, Pirow et al. (1999) demonstrated the importance of the feeding current for ventilation, and have shown that there is approximately 13 mmHg difference in oxygen partial pressure between the inflow and outflow of the feeding current associated with the ventral carapace. Given the dual role of the feeding current for feeding and ventilation, the priorities placed on feeding and ventilatory function vary in a complex manner that depends on food and oxygen availability, and this ultimately affects both ABR and relative importance of HR in oxyregulation (Paul et al., 1997; Pirow, 2004). Little is known about neurophysiological basis of feeding current regulation.

We have provided evidence that there is species-dependent cholinergic modulation of the rhythmic activity of the thoracic appendages by muscarinic-like and nicotinic-like receptors. If the cholinergic influences on ABR are centrally located, then these cholinergic mechanisms may play a critical modulatory role in the regulation of the feeding current. Likewise, the cholinergic modulation of the fast rotary tremor of the eye and the swimming antennae also seems quite significant and important for survival. Exposure to physostigmine and other AChE-Is that are used as insecticides (Zein et al., 2014) would cause overstimulation of cholinergic

receptors and would be clearly maladaptive. In the short time-course of these experiments, the muscarinic antagonist, atropine, did not have significant effects on ABR or HR. However, little is known about the long-term physiological impact of cholinergic receptor blockade on the ability to adapt to various stressors. The species-dependent effects of muscarinic receptor stimulation on HR (opposite) are particularly intriguing, and suggest some potential differences in the regulation of circulatory systems. Further pharmacological studies of neurotransmitter mechanisms may elucidate more species-dependent regulation of circulation and ventilation within the family, *Daphniidae.* Species dependent differences in regulation of circulation and ventilation may represent adaptations to differing habitats. For example, (Pirow et al., 2004) showed that competing influences on "the feeding current" can result in HR adjustments so that tissues receive an adequate oxygen supply. In *D. magna*, when food is plentiful (algae), ABR can be reduced to decrease filtration, but HR may increase to move hemolymph more rapidly through tissues.

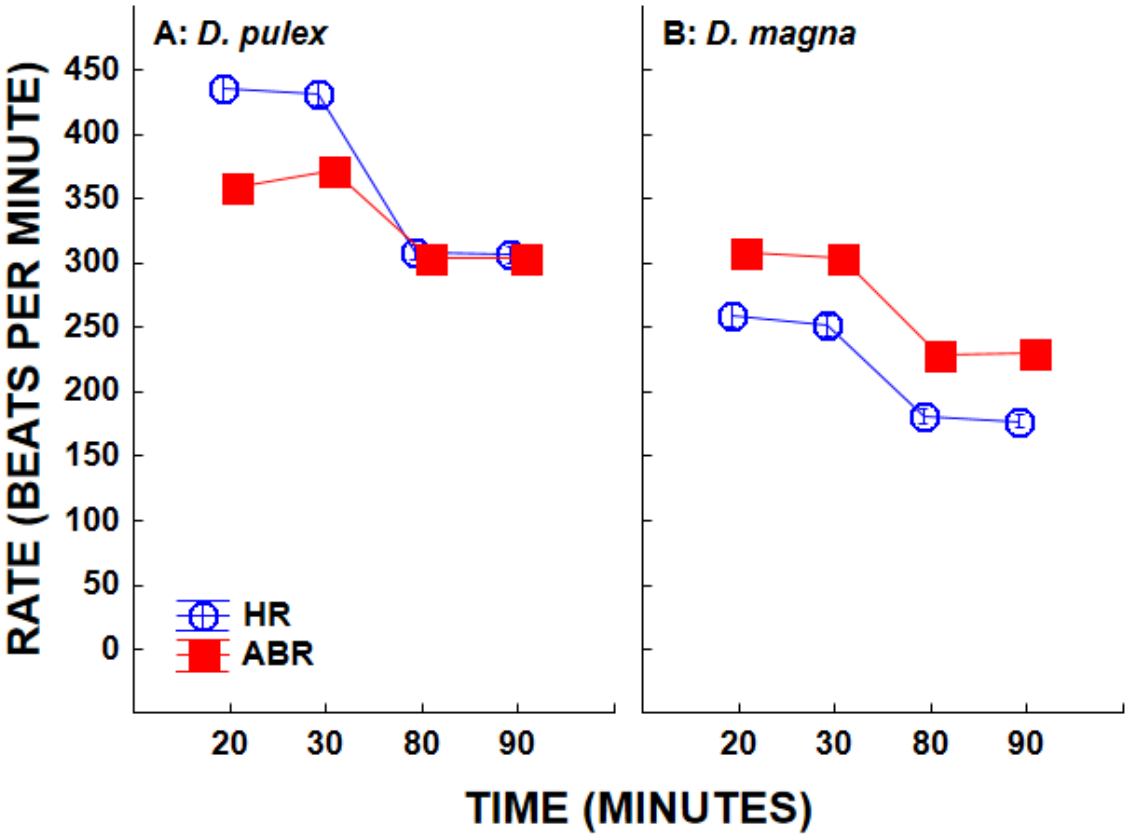

Figure 1 – Effect of temperature on *Daphnia pulex* and *Daphnia magna*

Time points were chosen to showcase the effect of temperature on *D. pulex* (left) and *D. magna* (right) HR and ABR. Temperature was lowered from 20ºC at 30 min to 15ºC for the remainder of the experiment.

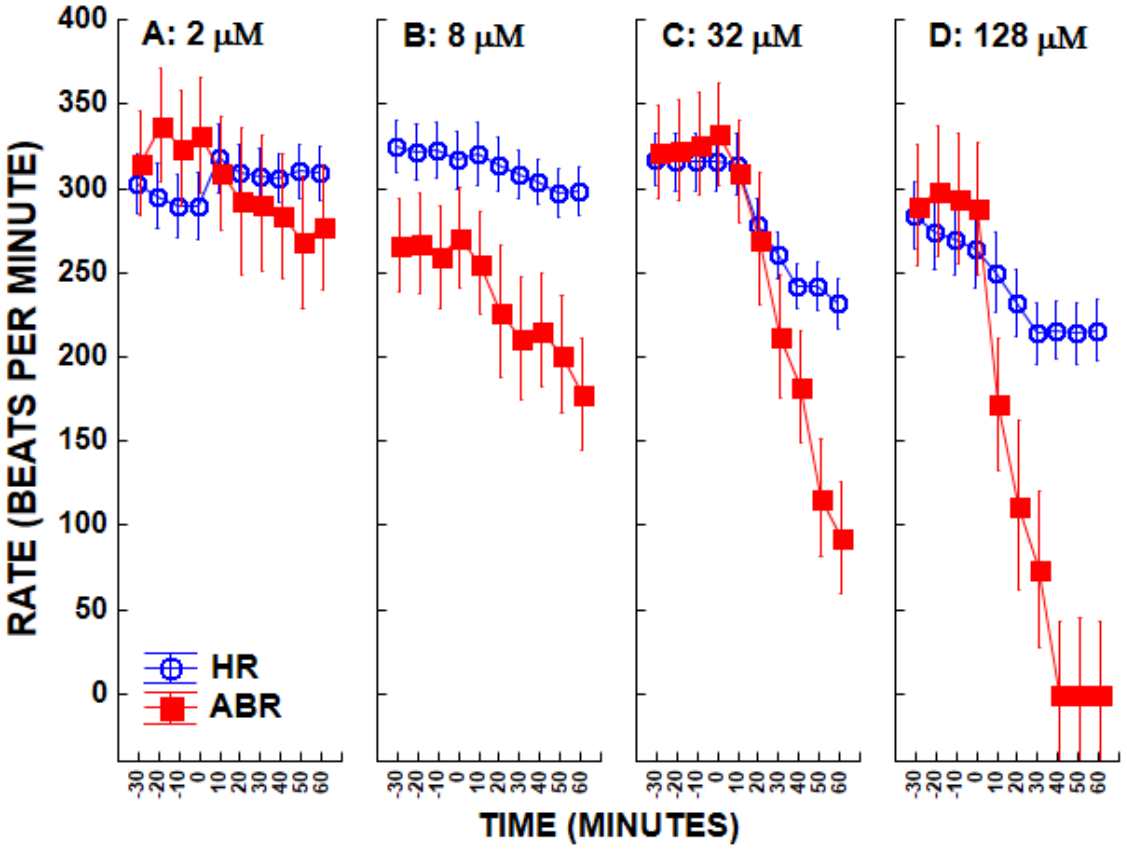

Figure 2 – Effect of Oxotremorine on *Daphnia pulex*

Effect of Oxotremorine (2 µm, n=4; 8 µm, n=5; 32 µm, n=5; 128 µm, n=3) on the HR and ABR of *D. pulex*. Error bars represent ± one standard error.

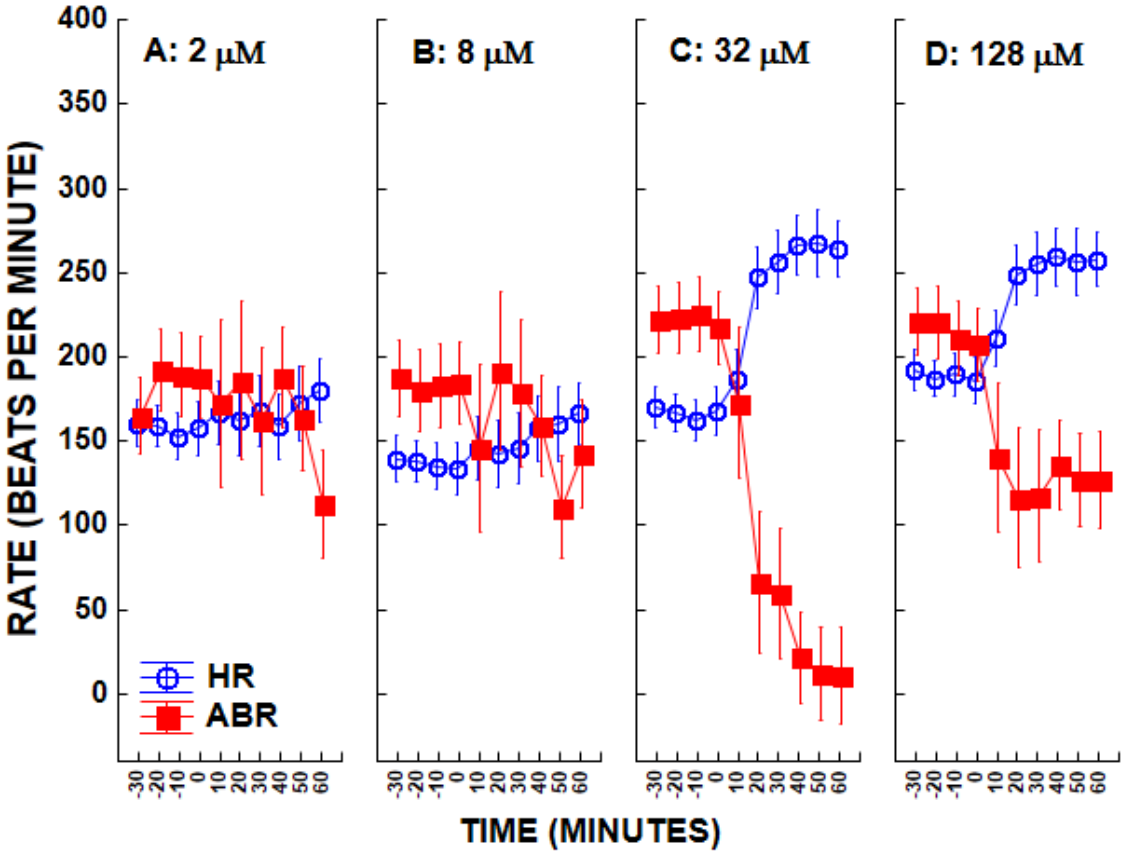

Figure 3 – Effect of Oxotremorine on *Daphnia magna*

Effect of Oxotremorine (2 µm, n=4; 8 µm, n=4; 32 µm, n=5; 128 µm, n=5) on the HR and ABR of *D. magna*. Error bars represent ± one standard error.

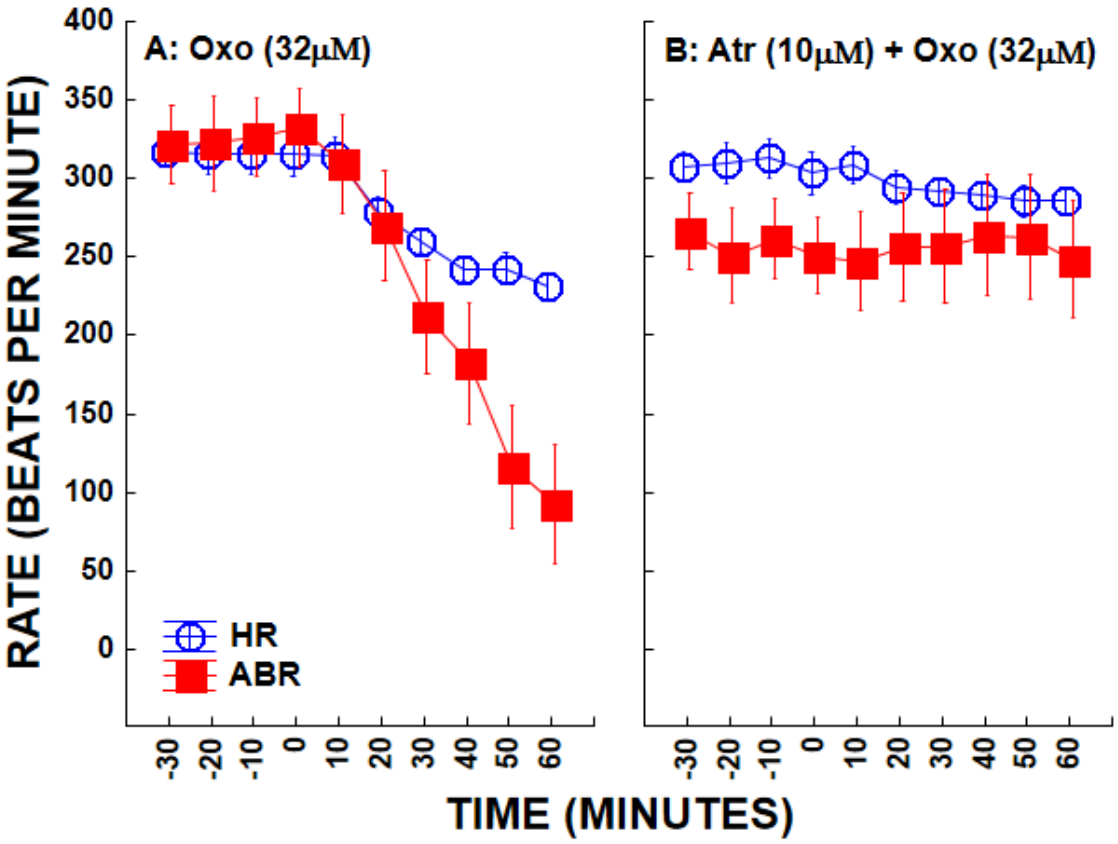

Figure 4 – Effect of Atropine pretreatment and Oxotremorine on *Daphnia pulex*

Panel A shows the effect oxotremorine (32 μm) (n=5) has on HR and ABR of *D. pulex*. Panel B, in comparison, showcases the effect an atropine pretreatment (10 μm) has on HR and ABR when followed by an atropine (10 μm) and oxotremorine (32 μm) combination (n=5). The (-20 min) time point in B marks introduction of atropine (10 μm) alone. The 0 time point in both marks the introduction of oxotremorine (A), and atropine –oxotremorine (B) solutions. All error bars are the standard error.

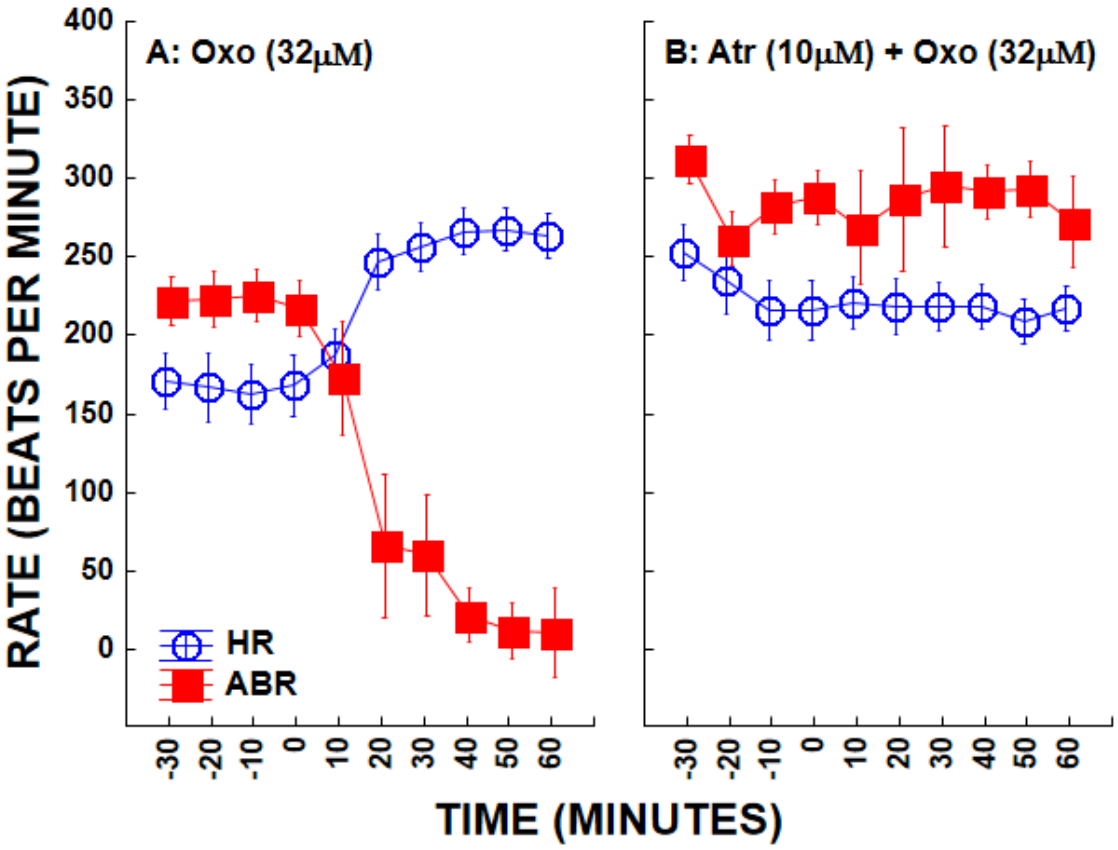

Figure 5 - Effect of Atropine pretreatment and Oxotremorine on *Daphnia magna*

Panel A shows the effect oxotremorine (32 μm, n=5) has on HR and ABR of *D. magna*. Panel B, in comparison, showcases the effect an atropine pretreatment (10 μm) has on HR and ABR when followed by an atropine (10 μm) and oxotremorine (32 μm) combination (n=5). The (-20 min) time point in B marks introduction of atropine (10 μm) alone. The 0 time point in both marks the introduction of oxotremorine (A), and atropine – oxotremorine (B) solutions. Error bars represent ± one standard error.

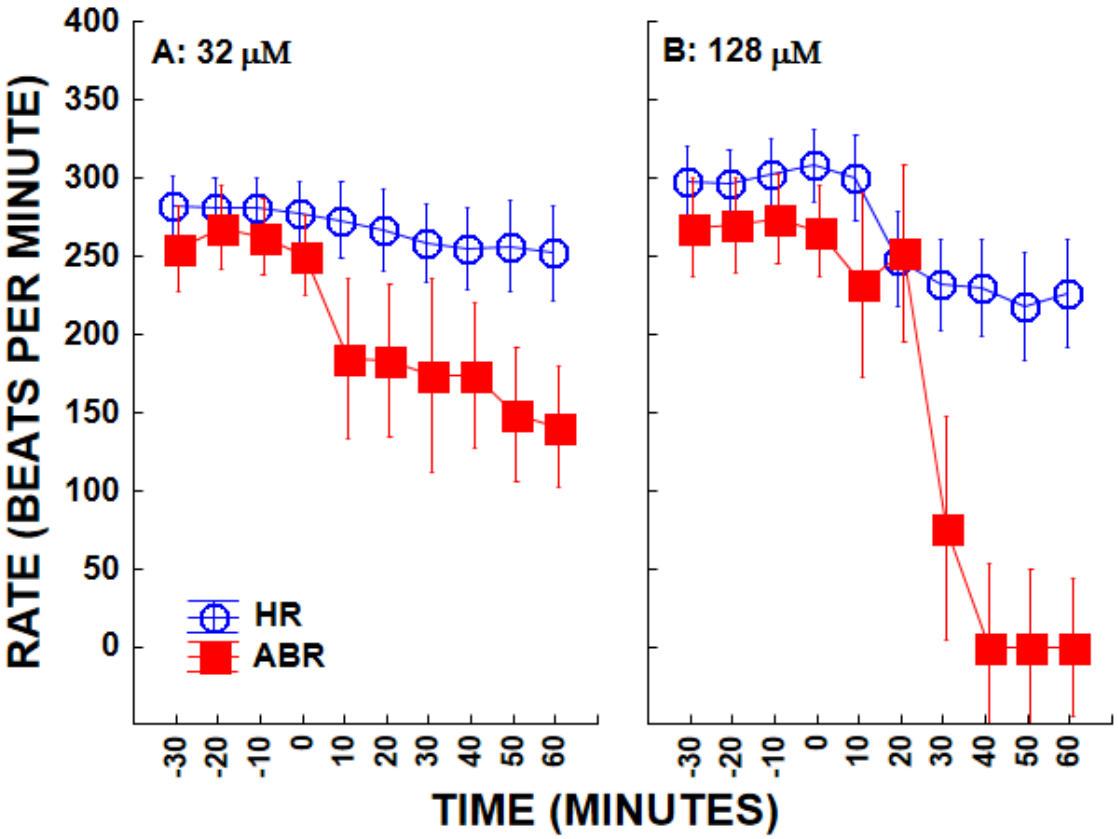

Figure 6 – Effect of Pilocarpine on *Daphnia pulex*

Effect of pilocarpine (32 µm, n=4; 128 µm, n=3) on the HR and ABR of *D. pulex*. Error bars represent ± one standard error.

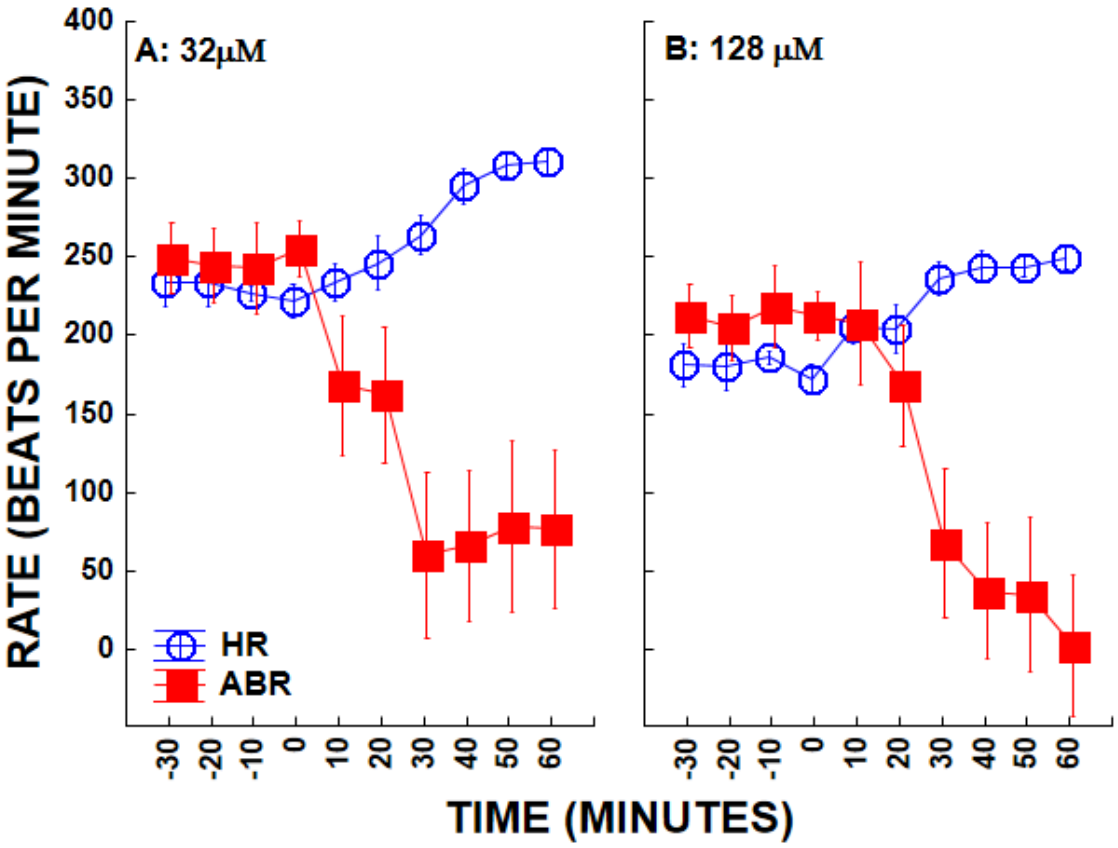

Figure 7 – Effect of Pilocarpine on *Daphnia magna*

Effect of pilocarpine (32 µm, n=4; 128 µm, n=5) on the HR and ABR of *D. magna*. Error bars represent ± one standard error.

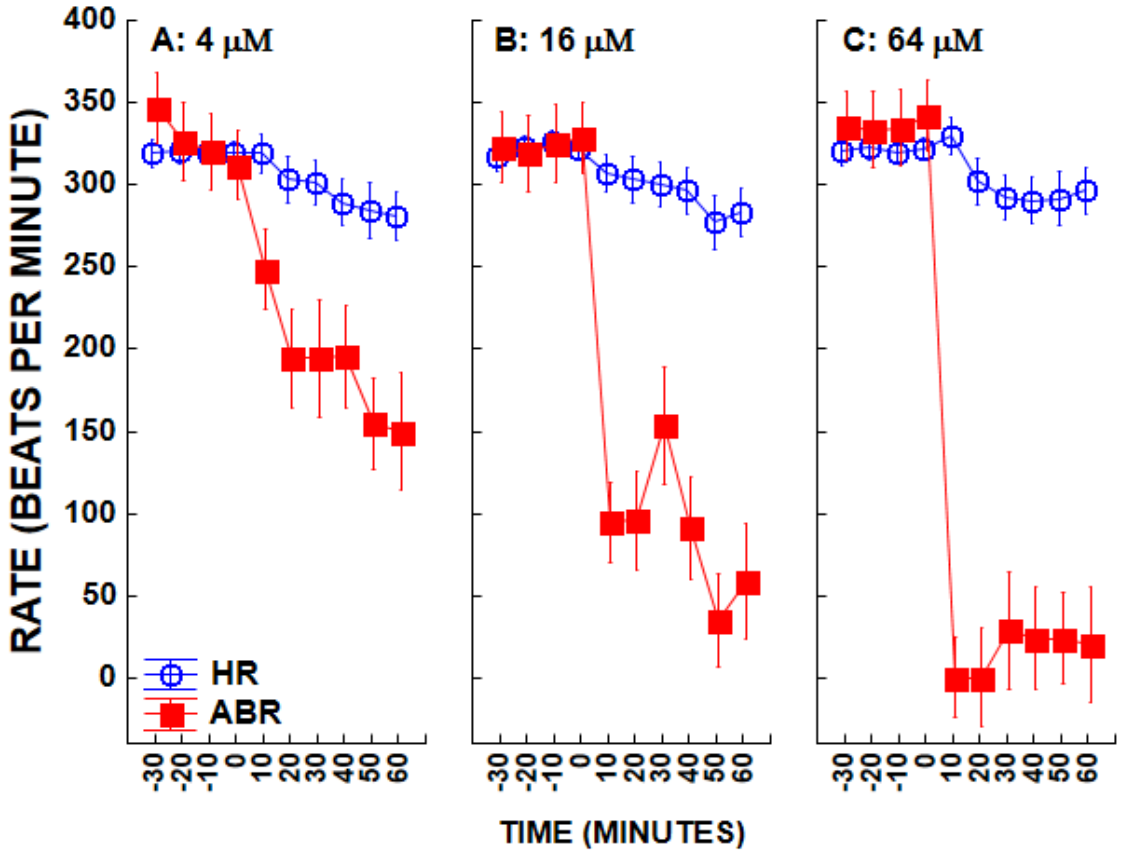

Figure 8 – Effect of Nicotine on *Daphnia pulex*

Effect of nicotine (4 µm, n=5; 16 µm, n=5; 64 µm, n=5) on the HR and ABR of *D. pulex*.

Error bars represent ± one standard error.

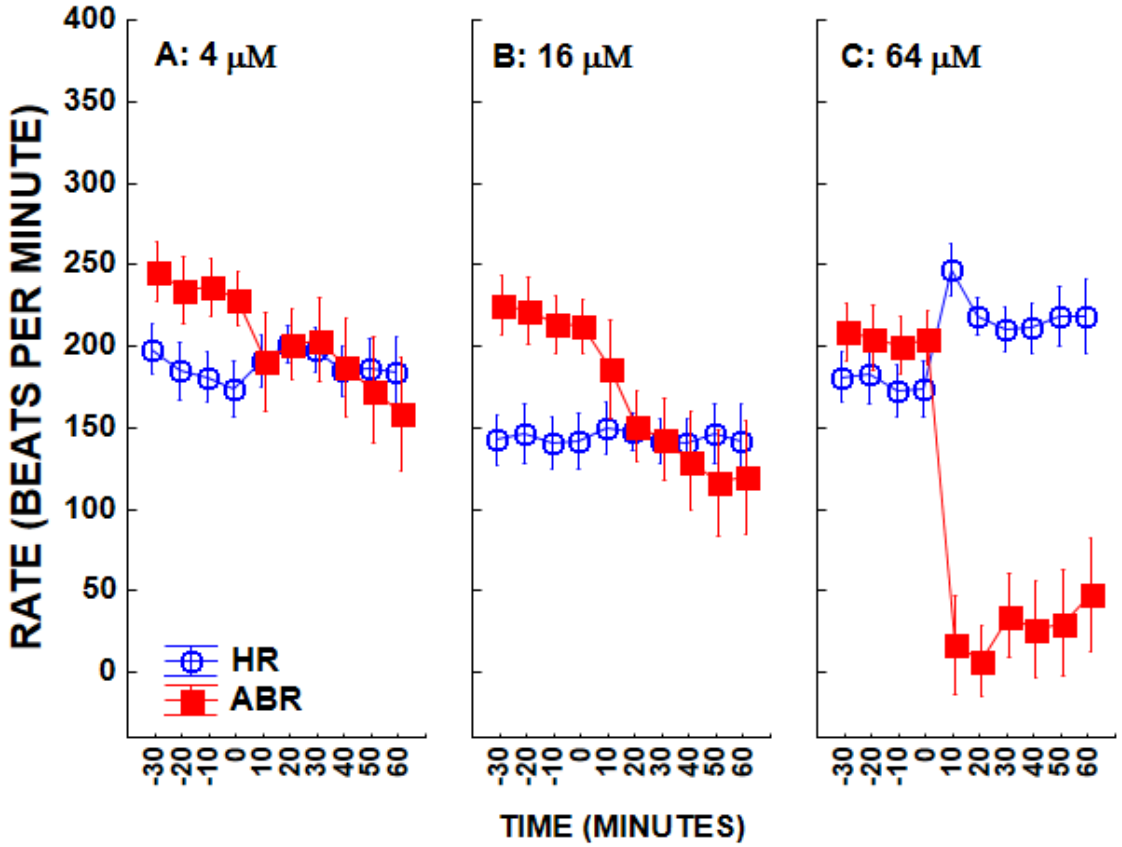

Figure 9 – Effect of Nicotine on *Daphnia magna*

Effect of nicotine (4µm, n=5; 16µm, n=5; 64µm, n=5) on the HR and ABR of *D. magna*.

Error bars represent ± one standard error.

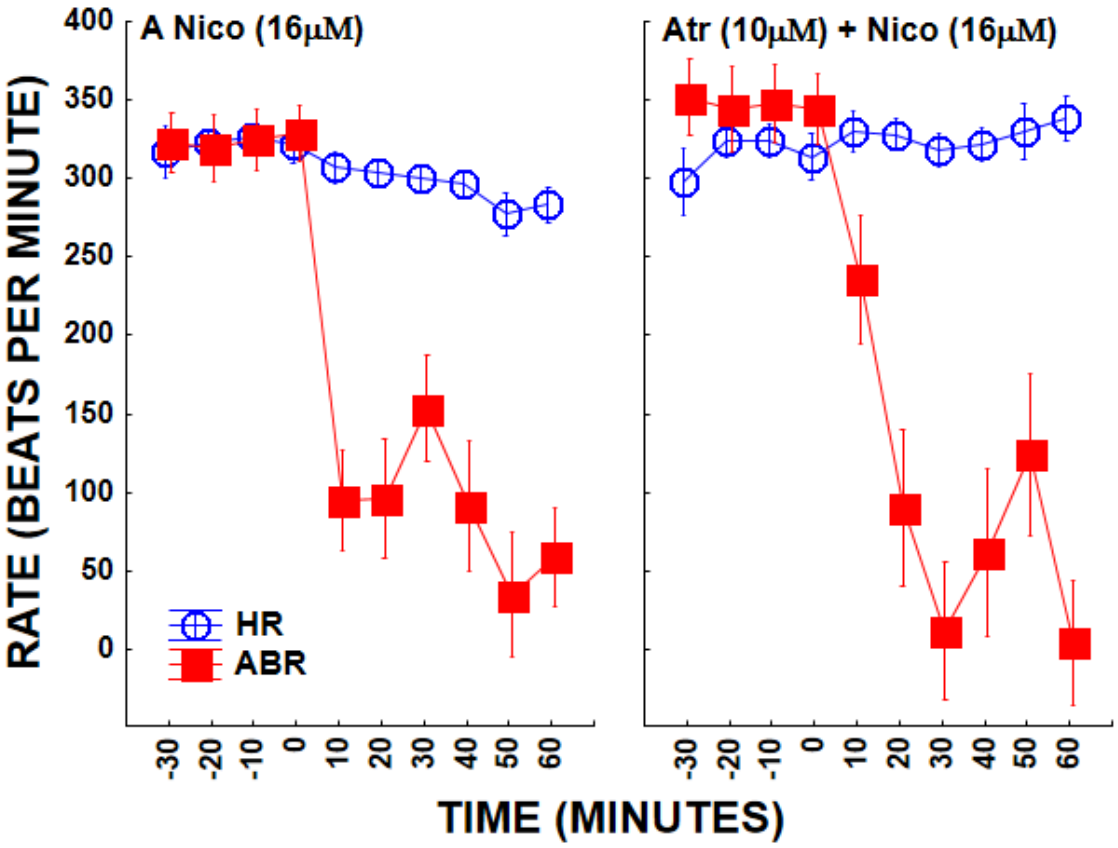

Figure 10 – Effect of Atropine Pretreatment and Nicotine on *Daphnia* pulex

Panel A shows the effect nicotine (16 µm; n=5) has on HR and ABR of *D. pulex*. Panel B, in comparison, showcases the effect an atropine pretreatment (10 µm) has on HR and ABR when followed by an atropine (10 µm) and nicotine (32 µm) combination (n=3). The (-20 min) time point in B marks introduction of atropine (10 µm) alone. Nicotine (A), and atropine-nicotine (B) solutions were introduced at t = 0 min. Error bars represent ± one standard error.

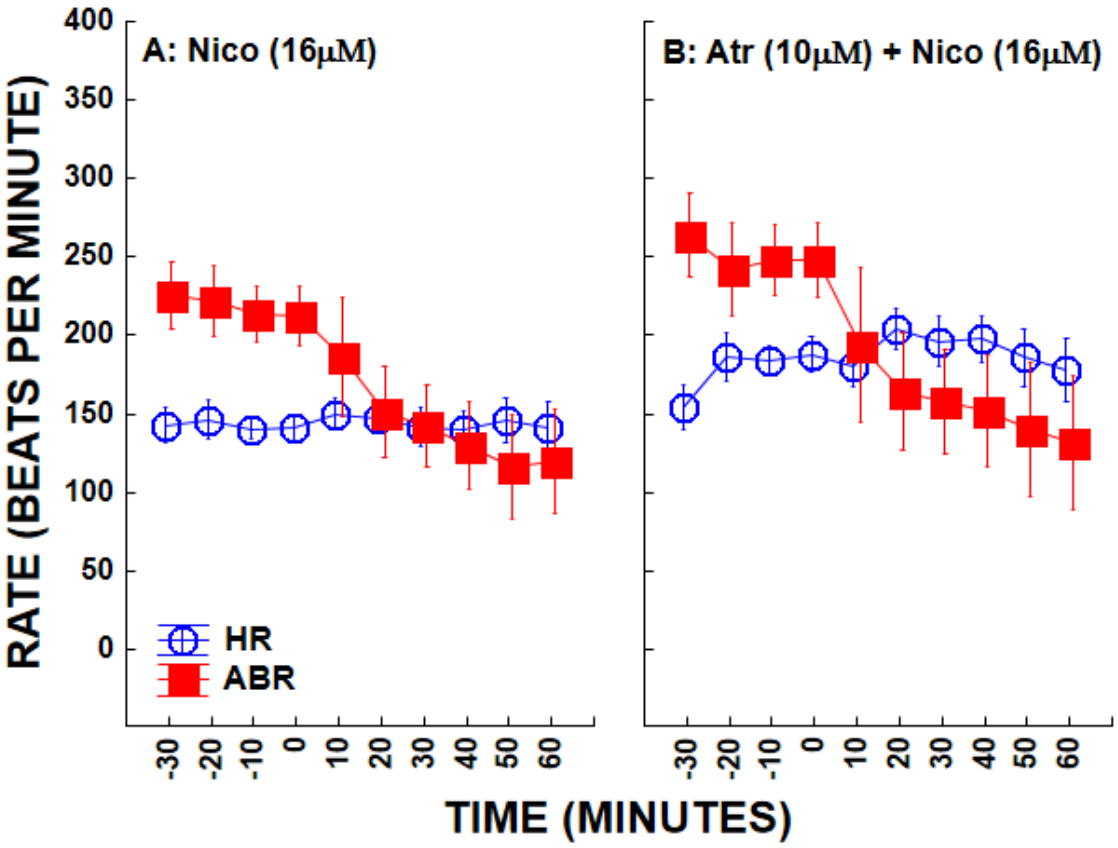

Figure 11 - Effect of Atropine Pretreatment and Nicotine on *Daphnia magna*

Panel A shows the effect nicotine (16 µm, n=5) has on HR and ABR of *D. magna*. Panel B, in comparison, showcases the effect an atropine pretreatment (10 µm) has on HR and ABR when followed by an atropine (10 µm) and Nicotine (32 µm) combination (n=3). The (-20 min) time point in B marks introduction of atropine (10 µm) alone. The 0 time point in both marks the introduction of nicotine (A), and atropine – nicotine (B) solutions. Error bars represent ± one standard error.

Table 1

Motor activity: Paralysis of the fast rotary tremor of the eye and activity of the swimming antennae (S.A.) by direct receptor agonists.

Agonist (concentration μM)	Eye - D. pulex % (n)	Eye - D. magna % (n)	S.A. - D. pulex % (n)	S.A. - D. magna % (n)
Oxo (2)	0 (4)	0 (4)	0 (4)	0 (4)
Oxo (8)	0 (5)	0 (4)	0 (5)	0 (4)
Oxo (32)	0 (5)	0 (5)	0 (5)	0 (5)
Oxo (128)	0 (3)	0 (5)	0 (3)	0 (5)
Oxo (32) + Atr (10)	0 (5)	0 (5)	0(5)	0 (5)
Nic (4)	0 (5)	0 (5)	0 (5)	0 (5)
Nic (16)	0 (5)	0 (5)	0 (5)	0 (5)
Nic (64)	0 (5)	0 (5)	0 (5)	20 (5)
Nic (16) + Atr (10)	0 (3)	0 (3)	0 (3)	0 (3)

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Abstract

SPECIES – DEPENDENT CARDIAC AND MOTOR RESPONSES TO CHOLINERGIC STIMULATION IN DAPHNIA PULEX AND DAPHNIA MAGNA

by

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Degree: Master of Science

The role of acetylcholine (ACh) in regulating the activity of the heart and 'feeding current' driven by the beating thoracic appendages of *Daphnia pulex* and *Daphnia magna* was evaluated using acetylcholinesterase inhibitors (AChE-I) and muscarinic receptor agonists. Single animals, tethered to a stainless steel pin, were tested in a watertight aquatic chamber that allowed free movement of appendages and swimming antennae. Heart contraction rate and the rate of thoracic appendage beating were quantified optically by measuring fluctuating changes in light-intensity caused by movement. Physostigmine, neostigmine, oxotremorine, pilocarpine as well as nicotine were used to study ACh and AChE. Atropine was used to attempt to block responses caused by the drugs used. The findings strongly support the role of ACh in the regulation of appendage beat rate, and suggest that AChE inhibition results in sufficiently high ACh levels to affect the rate of rhythmic appendage beating.

Autobiographical Statement

I was born and raised in the Detroit suburb of Warren, Michigan. My whole life I had a passion for the sciences, understanding "why and how come" were things I always needed to know. The support and encouragement of my high school science teachers, along with my passion for science and research, ultimately led me to Bowling Green State University in Bowling Green, Ohio. After four long years I completed my Bachelor's Degree in biology with a minor in chemistry. During my time there my interests went from marine biology to the pharmacological field and peaked with the connections that could be made between the two. This led me to begin researching graduate schools and advisors for whom I could work for who would let me further my knowledge and understanding in both fields. I ended up at Wayne State University where for the last 3 years I have been able to grow and learn in both areas of interest. The ability to connect pharmacology and drug mechanisms to my love for the aquatic life and natural environment have made my time in lab everything I have hoped it would be. I am truly blessed to have become part of a multidisciplinary team here at Wayne who have helped me expand my knowledge in biology and pharmacology, as well create a new passion in toxicology and environmental engineering. My research focused on understanding the anatomy and physiology of a crucial aquatic organism (Daphnia), which I truly believe will lead the way in future environmental toxicity studies.

As I look back at my time at Wayne, I am very thankful for all the people I have met and collaborated with. I am excited to graduate and begin the next chapter of my life where I am hopefully able to use all aspects of my past studies in my career.