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Effects of melatonin on hemolymph glucose and lactate concentrations in the fiddler crab, uca pugilator

Kathryn M. Dalton
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THE EFFECTS OF MELATONIN ON HEMOLYMPH GLUCOSE AND LACTATE CONCENTRATIONS IN THE FIDDLER CRAB, *UCA PUGILATOR*

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Thesis Presented to the Department of Chemistry,
Colby College, Waterville, ME
In Partial Fulfillment of the Requirements for Graduation
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Approved:

5-24-02 Date

Andrea R. Tilden, Ph.D.
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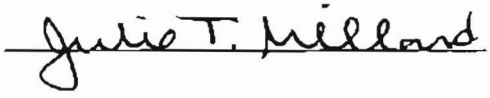

5/23/02 Date

Julie T. Millard, Ph.D.
Reader
Associate Professor of Chemistry

Submitted May 2002

One Moment In Time

Each day I live
I want to be
A day to give
The best of me
I'm only one but not alone
My finest day is yet unknown
I broke my heart for every gain
To taste the sweet I face the pain
I rise and fall
Yet through it all
This much remains

Chorus:
Give me one moment in time
(First chorus: I want one moment in time)
When I'm more than I thought I could be
When all of my dreams are a heartbeat away
And the answers are all up to me
Give me one moment in time
When I'm racing with destiny
Then in that one moment of time
I will feel, I will feel eternity
(Last chorus: I will be (2x), I will be free (2x))

I've lived to be the very best
I want it all, no time for less
I've laid the plans
Now lay the chance
Here in my hands

Chorus

You're a winner for a lifetime
If you seize that one moment in time
Make it shine

Chorus

Written by Albert Hammond and John Bettis
Empire Music Ltd./Warner Bros. Music Ltd.

To my mother
for being my biggest fan
and instilling in me
her own confidence,
independence,
wisdom
and compassion.

ABSTRACT

Melatonin (N-acetyl-5-methoxytryptamine) is an indolamine hormone produced by the pineal gland that works to regulate sleep/wake cycles and activity rhythms. The effects of melatonin in metabolism are far from understood. Melatonin was injected into the fiddler crab, *Uca pugilator*, to investigate the effects of melatonin on hemolymph glucose and lactate levels. Following injection at t=0, hemolymph samples were collected at t=0.5, 1.0, 1.5 and 5.0 hours. Melatonin caused a decrease in the stress response to injection and also caused delayed hyperglycemia. Melatonin-injected crabs also retained the glucose and lactate rhythymicity when compared to saline-injected crabs. Glucose and lactate rhythms followed the same pattern indicating that the cycles are coupled. Also, melatonin was synthesized using the Fischer Indole synthesis and characterized using H^1NMR. The synthetic melatonin demonstrated biological activity when injected into the crabs as when compared to pure melatonin on the effects on glucose and lactate concentrations. Overall, melatonin influences both glucose metabolism and the production of lactate.

VITAE

Kathryn Marie Dalton was born April 21, 1980 in Danvers, Massachusetts. She currently resides in Amesbury, Massachusetts with her moms, Celeste and Barbara, her brother, Matthew, along with 2 dogs, Lily and Abbey, and one cat, Pumpkin. Her father, Robert and stepmother Cindy, live in Lone Tree, Colorado with their stepson Matthew. (Yes, she has two brothers with the name Matthew)

Kathryn, known to her friends as "Dr. Dalts", graduated in 1998 from Timberlane Regional High School in Plaistow, NH with numerous accolades, including Honors with Distinction, French Honor Society, NH Women's Scholar Athlete Award, Science Achievement Award and National Honor Society. In addition to her academic achievements, Kate was a member of the varsity softball and volleyball teams, volunteered as a teacher's aide and tutor, and was active in the Plaistow Police Explorer organization. Her musical talents encompass playing the clarinet with the high school band, piano playing, and writing her own music.

Kate attended Colby College from 1998 through 2002. She studied abroad in France for her first semester of her freshman year, expanding her understanding of the French language and culture. Kate, a Chemistry major with a concentration in Cellular and Molecular Biology, anticipates graduating with honors in May. While attending Colby, Kate has been a "SCRUBS" pre-health professional leader, a Chemistry Teacher's Assistant and a tutor of Organic and General Chemistry. Her extracurricular activities include playing on the varsity volleyball team, competing with the diving team and leading the varsity softball team as a captain. Kate has recently completed Colby's EMT course, and she has volunteered her time as a member of the Colby Emergency Response Team and in the local Inland Hospital Emergency Room.

Kate's hobbies include diving, skiing, water skiing, white water kayaking, photography, jet skiing, traveling and having surgery (a.k.a. "grandma") Last summer, Kate broadened her horizons even further while volunteering in Africa with the "Intercultural Dimensions" organization. In Africa, Kate studied the educational and health care needs of the Senegalese people, and at the same time brushed up on her French and learned the native language of "Wolof". After graduation Kate is planning to work as an EMT while applying to medical school. Kate aspires to become a surgeon, where she can combine her many talents and compassionate nature in order to positively impact peoples' lives.

ACKNOWLEDGMENTS

I am grateful to Prof. Andrea Tilden for all of her help as my research and thesis advisor and with whom I have had the pleasure of working with over the past year.

I also wish to thank Dr. Julie Millard for generously giving her time to read my thesis.

I'd also like to thank Alexis Bond for keeping me company while injecting the crabs. It's pretty scary in the fourth floor lab of Arey all alone.

I owe Prof. Thamattoor and Prof. McIntyre for all of their help with the melatonin synthesis and by allowing me to become another number in the already full Ochem lab.

Kearney Shanahan is indebted to me for giving him half of my melatonin during the synthesis because he poured his down the drain, but I wish to thanks him for helping me with the NMR.

I hope that Coach Bailey and my teammates can forgive me for all of the practices and games I missed throughout the season to finish my research and thesis. I wish to thank them for their patience and support. Although, none of them showed up to my talk!!

Last but not least, I wish to acknowledge Prof. Mundy for his continuous inspiration and support while pursuing a major in chemistry. When I signed up to be a chemistry major, I wasn't too sure of what I was getting myself into. Prof. Mundy believed in me and told me I could do it. Although, I have not stood out as demonstrating stellar academic achievement, I have personally achieved so much by getting this far, and for that I owe to Prof. Mundy.

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INTRODUCTION

Melatonin (N-acetyl-5-methoxytryptamine, Figure 1) is an indolamine hormone produced by the photoreceptor cells of the retina and the pineal gland. The hormone exhibits a circadian rhythm resulting in low plasma daytime levels and high nighttime levels. Melatonin has been nicknamed the "darkness hormone" by acting as a time signal for an organism's daily (circadian) and annual (circannual) biological rhythms. The hormone works to regulate sleep/wake patterns, activity rhythms and synchronizes the release of other hormones.[1,2]

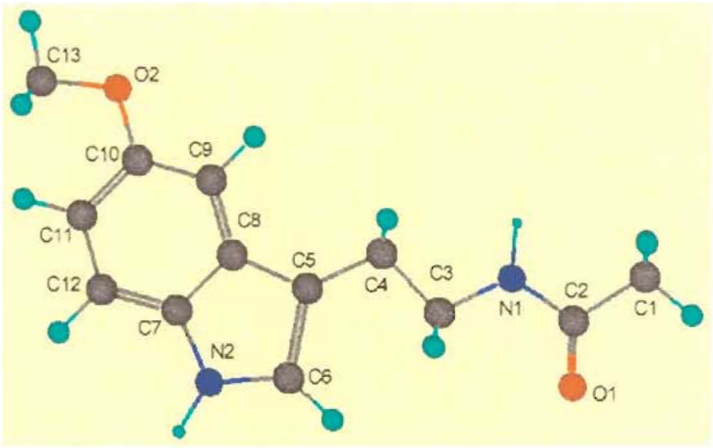

Figure 1: Structure of N-acetyl-5-methoxytryptamine (melatonin)

Melatonin was first isolated and characterized in 1958 from bovine pineal glands by Lerner and Case at Yale University.[3] Lerner discovered the light-related properties within the skin cells of amphibians. The molecule caused the aggregation of the pigment melanin within the melanocytes causing the skin to lighten. Because of its effects on melanin, Lerner named this new molecule "melatonin".[4]

In 1960, Weissbach *et al.* discovered the biosynthetic pathway of melatonin using rat liver and bovine pineal glands.[5] They found that melatonin is synthesized from serotonin through several biochemical reactions (Figure 2): (1) seratonin is converted to N-acetylserotonin by the enzyme serotonin N-acetyltransferase (AANAT) by addition of an acetyl group; (2) N-acetylserotonin is converted to melatonin by the enzyme hydroxyindole-O-methyltransferase (HIOMT), which methylates the hydroxyl group of the ring.[5,6] It was later discovered that seratonin and melatonin are both derivatives of the single amino acid tryptophan. Tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan hydroxylase. 5-HTP is converted to seratonin by decarboxylation by the enzyme aromatic amino acid decarboxylase.[5]

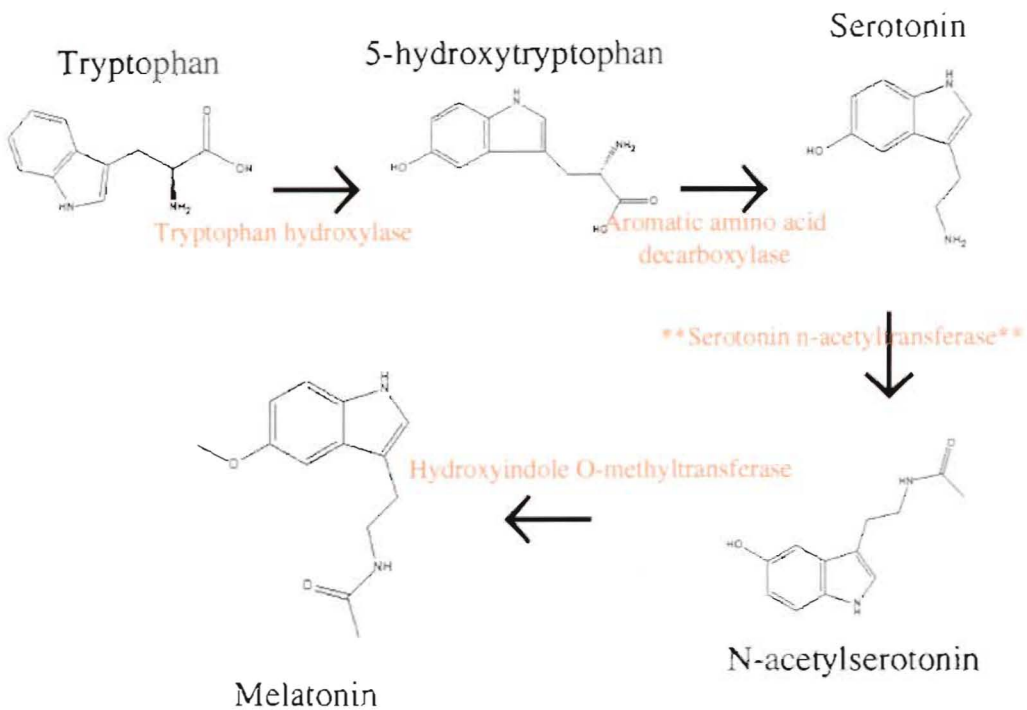

Figure 2: The biosynthetic pathway of melatonin

Seratonin N-acetyl transferase (AANAT) catalyzes the rate-limiting acetyl transfer from acetyl-coenzyme A to seratonin yielding N-acetylserotonin.[7,1,8] AANAT received a lot of attention after it was found to exhibit a large day/night rhythm. Itoh *et al.* discovered AANAT day/night rhythm in the hemolymph of the silkworm *Bombyx mori*.[1] AANAT demonstrates highest activities during the night and lowest activities during the day. Melatonin shows these same rhythmic changes. Less melatonin is produced during the day during low AANAT levels. More AANAT is available at night causing an increase in melatonin production. This led researchers to believe that melatonin synthesis is dependent on AANAT activity and there is some sort of photic depression of AANAT. Light exposure somehow inhibits acetylation and synthesis of melatonin.

The enzyme AANAT is under tight regulation by cAMP.[8] Cyclic AMP activates AANAT as well as its transcription and translation. Sympathetic innervations cause the release of norepinephrine, which binds to β-adrenoreceptors and stimulates adenylate cyclase activity. The cAMP activates cAMP-dependent protein kinase A (PKA), which targets transcription factors in the nucleus and triggers AANAT gene expression. An adaptation to different photoperiods may be associated with a change in AANAT gene expression.[6] A dark-adapted organism would produce more of the enzyme AANAT to synthesize melatonin in response to darkness and therefore have an increase in AANAT gene expression. However, it is unclear whether gene expression is under post-translational regulation. Because AANAT is the rate-limiting step of melatonin biosynthesis, the enzyme is highly regulated. It may be a valuable target for developing inhibitors to melatonin synthesis to determine the physiological and pathophysiological

roles of melatonin.[9] Also it may be possible to examine mutations in the AANAT gene and the effects on melatonin synthesis.

The methoxy indolamine hormone has been discovered among vertebrates and invertebrates and has a plethora of functions. Melatonin is produced primarily by the pineal gland (a pea-sized organ in the brain) and also within the photoreceptors of the retina. The hormone within the retina acts locally to synchronize a local retinal clock. It inhibits the release of dopamine and acetylcholine from the amacrine cells during darkness and stimulates the release of glutamate.[10] The rhythmic pattern of melatonin synthesis and release by the pineal gland is under the control of hypothalamic suprachiasmatic nuclei (SCN) responding to the light/dark environment. The light /dark signals are transmitted from the retina to the SCN via the retino-hypothalamic tract and then sent out to the pineal gland by postganglionic sympathetic fibers.[11] Melatonin produced by the pineal gland is released into the organism's plasma or hemolymph comprising most of the circulating melatonin. The hormone is highly lipid soluble and is readily absorbed across cell membranes to penetrate the tissue. [4]

The rhythmic expression contributes to the time-giving properties of melatonin. This suggests that melatonin may have potential therapeutic applications, such as to resynchronize body rhythms and sleep/wake cycles. Research is currently being conducted to assess the implications of melatonin administration on human systems. Minors and Waterhouse discovered disturbances in sleep and mood in night-shift workers due to disrupted body rhythms, for which melatonin could be a potential treatment.[12]

Due to the United States Dietary and Supplement Health and Education Act, herbs, vitamins, minerals and amino acids are no longer under the regulation of the Food and Drug Administration (FDA). Because melatonin is found in trace amounts in foods

such as bananas and rice, the compound can be sold over the counter. At least 10 million people have taken melatonin as a dietary supplement and sales have been as high as 200 million dollars.[4] With all of the hype, people began taking melatonin for a variety of ailments and claims. For most claims such as to treat sexual dysfunction, neurological disorders, immune disorders, longer life and arthritis, melatonin doesn't work. However, the hormone is being extensively studied for treating sleep disorders, daily rhythms, jet lag and some diseases due to a synergy with pyridoxine (vitamin B_6).[13]

The study by Arendt *et al.* at the University of Surry was the first to investigate the effect of daily-administered melatonin on human biological rhythms.[14] They administered 2 mg of melatonin daily at 1700 h for one month. They found that when compared with a placebo, melatonin produced an advance in its own rhythm, advance in the timing of the hormone prolactin and an advance in the onset of tiredness.

Lewy *et al.* of the Oregon Health Sciences University showed that melatonin had an effect on the daily rhythms according to a phase-response curve.[15] They administered 0.5 mg of melatonin for four days. They found that when melatonin was administered in the morning, the daily rhythms were advanced and when administered in the evening, the rhythms were delayed. Claustrat *et al.* of Lyon, France, also discovered phase response curves for melatonin injections.[16] The advance or delay of the rhythm depends on the time of injection.

The Federal Aviation Administration (FAA) and the Aerospace Medical Association are both expressing interest in melatonin for use by aviation workers and air travelers to treat jet lag.[11] International passengers and crew and nighttime flyers are susceptible to circadian desynchronization. Harma *et al.* found disruption in salivary melatonin levels of aircrew from transmeridian flights.[17] Arendt *et al.* found that

administering 5 mg of melatonin three days before flight and four days after improves sleep quality and mood.[18] It reduces time taken to fall asleep, daytime alertness and resynchronization with cortisol levels as compared to a placebo.

All of these studies suggest that melatonin can potentially treat sleep disorders and resynchronize phase shifts. However, more clinical trials and research are needed. It is important to disclose the time of administration for optimal results depending on the phase of the body clock. It is also important to research potential drug interactions with melatonin as there is very little toxicological data. In addition, because melatonin is a relatively new dietary supplement, it is important to investigate the long-term effects of melatonin administrations. Future research may also involve developing melatonin analogues and antagonists.

Clinical studies on animals have provided insight into the functions of melatonin and are the basis of further study for human applications. The regulation and biosynthesis of melatonin is similar all living organisms that have a day-night rhythm of hormonal oscillations. Invertebrates are useful model organisms because they have simple nervous systems. There are few surgical constraints and researchers can use large sample sizes. The use of a model organism allows researchers to examine the individual biosynthetic pathways and the molecular and physiological mechanisms of melatonin production.

The fiddler crab (family Ocypodidae and genus Uca), is extensively used in research to study crustacean hormones and biological rhythms.[19] The fiddler crab is a choice organism for the study of melatonin because they are available in large sample sizes, affordable and easy to care for. There are few constraints to surgical procedures and they are notably resilient.

Figure 3: The Fiddler Crab, *Uca pugilator*

The crabs are easily recognized by their square body with a marked difference in the size of the right and left claws of the males. They are common along the southeastern Atlantic coast of sandy or marsh habitats. They are one of the most conspicuous inhabitants of the shores at low tides since they are often found foraging in groups of thousands and millions. They feed on small organic matter and are most active feeders at dawn and dusk. The fiddler is most active during spring tides. They follow a diurnal courtship that involves physical and acoustic signals. The male stomps his walking legs and drums his larger claw on the sand. Because of their aggressive behavior and combat between competing males, the *Uca pugilator* is also know as the "fighting fiddler". Tilden *et al.* discovered a circadian rhythm of melatonin in the eyestalks of the *Uca pugilator* under conditions of light and darkness, suggesting that melatonin carries photoperiodic information.[20]

The purpose of this study was to investigate the effects of exogenous melatonin on glucose and lactate concentrations in the fiddler crab, *Uca pugilator*. Because melatonin has been shown to affect day/night rhythms, it is hypothesized that melatonin affects glucose metabolism. Studies suggest that exogenous melatonin affects glucose metabolism. Tilden *et al.* found that melatonin-injected fiddler crabs demonstrate a delayed hyperglycemic response compared to saline injected crabs.[21] Another study by

Fleur *et al.* found that melatonin enhances the dark signal of the biological clock by decreasing glucose concentrations in the blood plasma of rats during the dark period.[22] In order to be active, the fiddler crabs must metabolize glucose. High hemolymph glucose levels should be highest during times of activity as a result of the mobilization of glucose to meet the energy requirement. Lactate levels should also cycle with glucose levels, as lactate is a product of anaerobic glucose metabolism during high activity.

METHODS AND MATERIALS

Melatonin Injection and Hemolymph Collection at 5 hours

Male intermolt fiddler crabs, *Uca pugilator* (Gulf Specimen Marine Laboratories, Inc., Panacea, FL) were divided into three clear plastic tanks that were tilted to allow the crabs to move in and out of the water. The crabs were fed wet cat food (Fancy Feast Ocean Blend ®) every other day and the seawater was changed the day after feeding. The crabs were kept at room temperature and acclimated to a 12:12 photoperiod, lights on at 0800h and turned off at 2000h.[21]

On the day of melatonin injection, one tank of crabs was injected with 100 µl of melatonin (0.05g/L) in seawater at 1130h at the base of the 3rd right walking leg. A second tank of crabs were injected with 100 µl of seawater.[21] The crabs in the third tank were not injected. After 5 hours, hemolymph samples were taken from the base of the 3rd right walking leg from groups: **MEL** (melatonin-injected crabs), **SEA** (seawater-injected crabs) and **NON** (non-injected crabs). The samples were immediately placed in the freezer at -4°C until assay.

The day of assay, the hemolymph samples were thawed and the glucose and lactate levels were measured using a YSI 2300 STAT Plus glucose and lactate analyzer (YSI, Yellow Springs, OH). The analyzer was autocalibrated to known concentrations of glucose and lactate before assay and after every 5 samples.

The glucose and lactate samples were compared within each group using a one way variance analysis (ANOVA) with a Student-Newman-Keuls test and the means were compared using a Student's T-test. Both statistical tests were completed using SigmaStat software (Jandel Corporation).

Synthesis of Melatonin

Melatonin is widely researched for different biological effects, such as sleep patterns, cancer, aging, depression, reproduction, jet lag and as an antioxidant. It is effective to have a simple and efficient method for melatonin synthesis to produce biologically active melatonin.

A simple and efficient method of melatonin synthesis is a one-pot Fischer Indole synthesis that uses 4-aminobutyraldehyde dimethylacetal as the starting product (Figure 4).[23] In 250 mL flask containing 2 mL of 1.08 g/mL acetic anhydride (Ac_2O), 2.5g of 4-aminobutryaldehyde dimethylacetal (**1**) was added over a 5 minute period while stirring on a 5-10 °C ice-water bath. The mixture was allowed to stir over the ice bath for 1 hour at the same temperature. After 1 hour, the flask was removed from the ice bath and 35 mL of acetic acid, 49 mL of ethanol and 56 mL of dH_2O were added ($AcOH/EtOH/H_2O$=2.5/3.5/4) to the intermediate (**2**). The mixture was allowed to stir for 10 minutes and 3.67 g of 4-methoxyphenylhydrazine hydrochloride salt was added. The mixture stirred at 40 °C for 12 hours. The mixture was concentrated on a rotovap at

40 °C and the concentrate was diluted with 20 mL of dH_2O. To the mixture, 10 mL of 0.5N-HCL was added and extracted three times with 20 mL of ethyl acetate. The extracts were combined and washed three times with 10 mL of saturated $NaCHO_3$ and then once with brine. The mixture was dried over anhydrous sodium sulfate ($NaSO_4$). Once dry, the mixture was filtered through a short silica gel bed and concentrated on a rotovap, which yielded the crude product. The crude product (**3**) was purified and recrystallized by washing with hot toluene and by decanting the toluene layer. The toluene fractions were combined and placed in ice to yield light orange crystals. The crystals were obtained by vacuum filtration. The pure product was analyzed using melting point analysis and H^1 NMR.

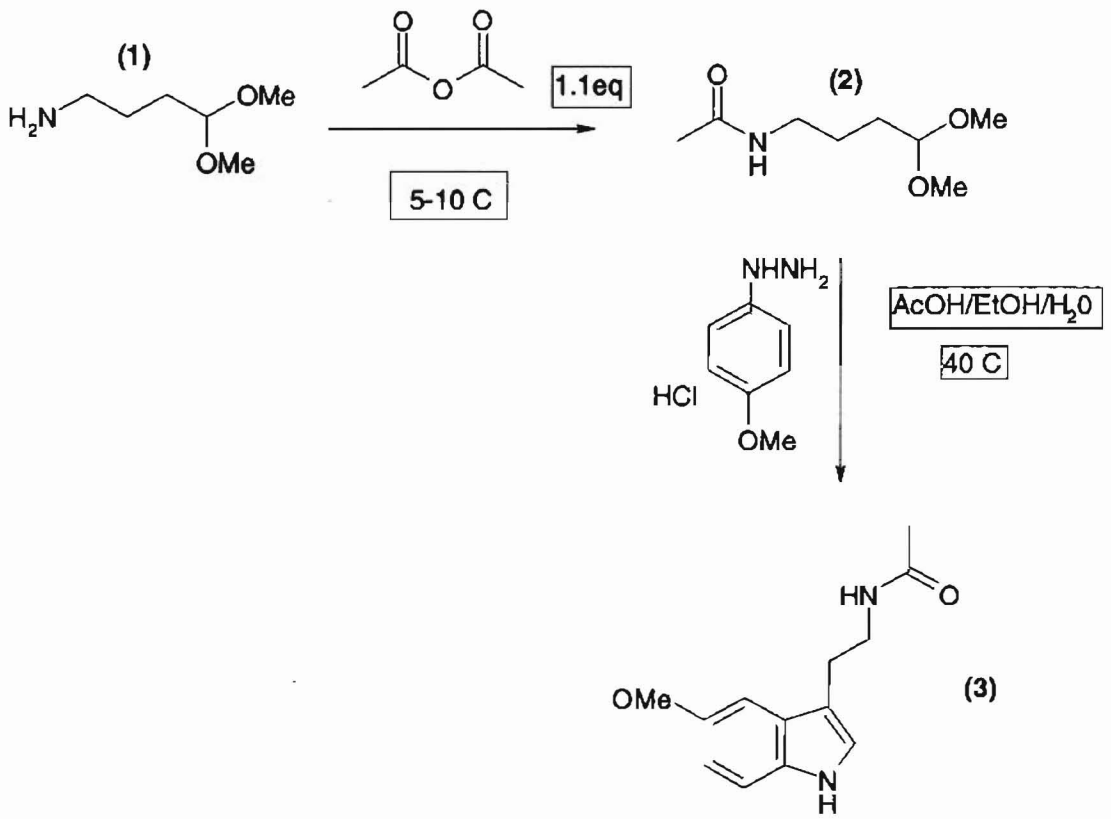

Figure 4: Fischer Indole synthesis of melatonin

Melatonin Injection and Collections of Hemolymph at 0.5h, 1.0h and 1.5h

To investigate the effects of melatonin injection on glucose and lactate levels immediately after injection, the fiddler crabs were injected at 1130h and hemolymph samples were collected 0.5h after injection. Hemolymph samples were also collected 1.0h and 1.5h. after injection. On the day of injection at 1130h, 45 crabs were injected with 100 µL of seawater and 45 were injected with 100 µL of seawater with 50 µg/mL melatonin at the base of the 3rd right walking leg. After 0.5, 1.0, 1.5 hours, hemolymph samples were taken from one of the left walking legs from 15 seawater-injected crabs (**SEA**), 15 melatonin-injected crabs (**MEL**) and 15 non-injected crabs (**NON**). The hemolymph samples were frozen immediately after extraction until the day of assay. Glucose and lactate concentrations were analyzed using the YS1 2300 STAT Plus glucose and lactate analyzer (YSI, Yellow Springs, OH)

Glucose and lactate samples were compared within each group using a one-way variance analysis (ANOVA) with a Student-Newman-Keuls test and the means were compared using a Student's T-test. Both statistical tests were completed using SigmaStat software (Jandel Corporation).

Synthesized Melatonin vs. Pure Melatonin: Injection of Synthetic Melatonin and Collection of Hemolymph Samples

Fiddler crabs were injected with synthesized melatonin to determine if the synthesized melatonin was biologically active. Hemolymph samples were collected and compared to the hemolymph samples obtained from pure melatonin-injected crabs and seawater-injected crabs.

On the day of injection, three groups of 15 crabs were injected with either 100 μL of 50 μg/mL synthetic melatonin, 100 μL of 50 μg/mL pure melatonin, or 100 μL of seawater at the base of the third walking leg. After three hours, hemolymph samples were collected and kept frozen until the day of assay. Glucose and lactate concentrations were analyzed using the YSI 2300 STAT Plus glucose and lactate analyzer.

Glucose and lactate samples were compared within each group using a one-way variance analysis (ANOVA) with a Student-Newman-Keuls test and the means were compared using a Student's T-test. Both statistical tests were completed using SigmaStat software (Jandel Corporation).

RESULTS

We used the YSI 2300 STAT Plus Glucose and Lactate Analyzer to Test Hemolymph Glucose and Lactate Levels

The YSI 2300 STAT plus glucose and lactate analyzer (Figure 5) for these studies is a fast and accurate method for analyzing glucose and lactate levels in the blood, plasma, serum and cerebrospinal fluid. It is used for clinical diagnostic work within the emergency room, stat labs, endocrinology/diabetes labs, pediatric/neonatal labs and others. It only requires 25 μL of sample and samples do not require dilution because the YSI measures up to 900 mg/dL of glucose and 30 mmol/L of lactate.

Figure 5: The YSI 2300 STAT Plus Glucose and Lactate Analyzer (Yellow Springs Inc.)

The YSI glucose and lactate analyzer is based on biosensor technology that uses enzyme specific membranes to target a particular substrate. The YSI has two membranes, one targets glucose and the other targets lactate. The membranes contain three layers, the polycarbonate layer, immobilized enzyme layer and the cellulose acetate layer (Figure 6).

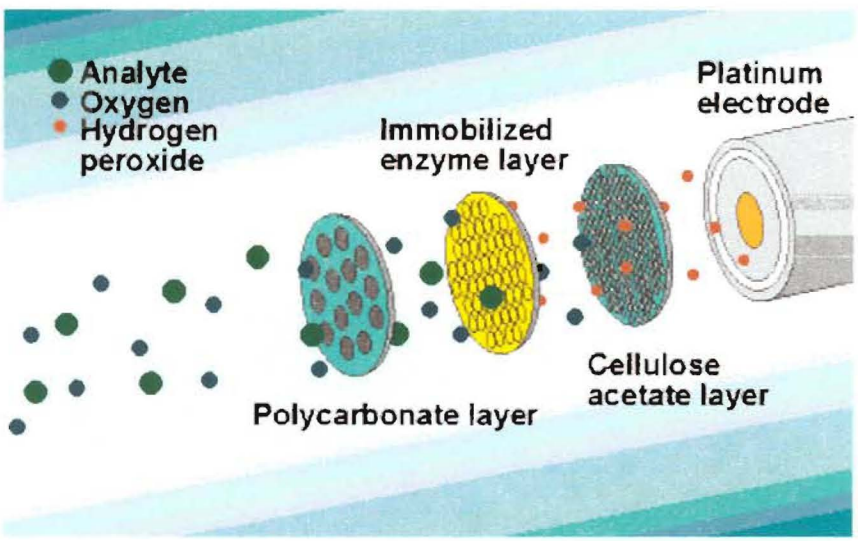

Figure 6: The YSI glucose and lactate analyzer three layered membrane using the biosensor technology

The porous polycarbonate layer limits diffusion of glucose and lactate to the immobilized enzyme layer preventing an enzyme limiting reaction. The immobilized enzyme layer holds glucose and lactate oxidase, which oxidizes D-glucose and L-lactate to produce hydrogen peroxide (Figures 7 and 8).

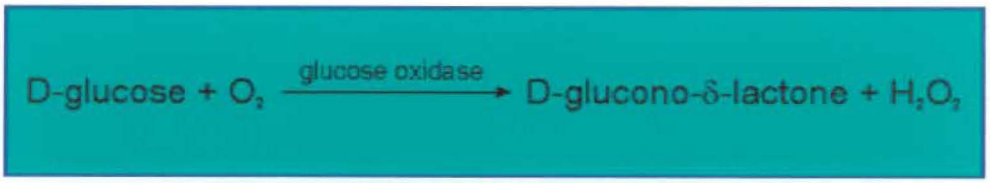

Figure 7:YSI glucose measurement: D-glucose is oxidized via the enzyme glucose oxidase producing glucono-lactone and hydrogen peroxide

Figure 8:YSI lactate measurement: L-lactate is oxidized via the enzyme L-lactate oxidase producing pyruvate and hydrogen peroxide

The cellulose and acetate layer only allows small molecules (MW<200), including hydrogen peroxide, to diffuse through to the platinum electrode, which oxidizes hydrogen peroxide, releasing electrons (Figure 9).

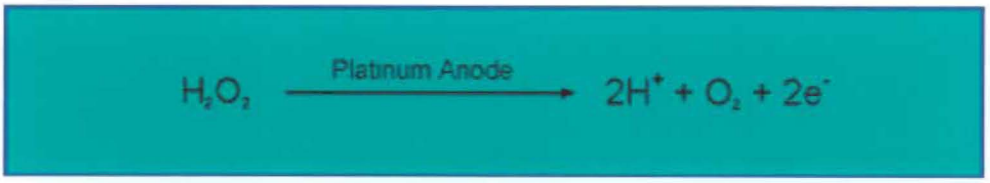

Figure 9: YSI hydrogen peroxide measurement: hydrogen peroxide oxidized at the platinum electrode producing electrons

The electrode measures the electron flow, and the electrons produced from the oxidation of hydrogen peroxide are proportional to the amount of glucose and lactate oxidized in a given sample.

Assay of Hemolymph Samples Collected 5 Hours After Melatonin Injection

Hemolymph samples were collected from melatonin-injected crabs, saline-injected crabs and non-injected crabs five hours post-injection of melatonin. Concentrations of metabolites within hemolymph samples were compared between groups. Lactate levels in melatonin-injected crabs (**MEL**) were significantly lower than non-injected crabs (**NON**) (Appendix A: Figure 10, $P<.001$). Seawater injected five hours before sampling caused a suppression in the hemolymph lactate concentration. Overall, lactate levels were lower in the seawater-injected crabs (**SEA**) than both the melatonin-injected (**MEL**) and the non-injected crabs (**NON**) ($P<.001$).

Melatonin also significantly decreased hemolymph glucose levels five hours post-injection of melatonin (Appendix A: Figure 11, $P<.001$). Seawater-injected crabs (**SEA**) showed a significant suppression in glucose levels ($P<.001$). The glucose concentrations of the seawater-injected crabs were much lower than the melatonin-injected crabs ($P<.001$). Melatonin caused a suppression of glucose levels five hours post-injection following the same pattern as lactate. Seawater injection caused the greatest response by significantly decreasing glucose and lactate concentrations.

Assay of Hemolymph Samples Collected at 0.5, 1.0 and 1.5 Hours After Melatonin Injection

In order to determine the effects of melatonin on the initial concentrations of glucose and lactate, hemolymph samples were assayed 0.5, 1.0 and 1.5 hours post-injection. The glucose and lactate levels were compared with the levels assayed 5 hours post-injection. A trend was established to determine why the seawater-injected crabs showed a significant suppression in the amount of hemolymph glucose and lactate levels.

Melatonin injection caused a significant initial suppression of hemolymph lactate concentrations and then an increase at five hours post-injection (Appendix A: Figure 12, $P<.001$). The seawater-injected crabs (**SEA**) also showed an initial suppression of lactate levels ($P<.001$) and the levels continued to decrease up to five hours post-injection. The non-injected crabs (**NON**) show the cycle of lactate concentrations over time, with decreasing concentrations until 1.5 hours and then increasing concentrations to 5 hours. The hemolymph lactate concentrations of melatonin-injected crabs (**MEL**) followed a similar pattern as the levels of the non-injected crabs. The seawater-injected crabs did not follow the same pattern by showing a suppressed lactate peak at 5 hours post-injection.

The melatonin-injected crabs (**MEL**) and non-injected crabs (**NON**) both demonstrated significantly lower hemolymph glucose concentrations than seawater-injected crabs (**SEA**) (Appendix A: Figure 13, $P<.001$). The glucose levels in melatonin-injected crabs follow the same pattern as non-injected crabs, with a peak at 5 hours post-injection. Seawater-injected crabs had increased initial glucose concentrations and had a significantly suppressed peak at 5 hours post-injection ($P<.001$).

Synthesis and Characterization of Melatonin

A light yellow solid was recovered from the melatonin synthesis. The product was characterized using melting point analysis and H^1 NMR which suggested that the proposed melatonin synthesis did in fact yield melatonin. From 2.5 g of 4-aminibutyraldehyde (133.19 g/mol), 0.717 g of melatonin was recovered (232 g/mol) at a 17% yield. The melting point of the product was 112.5-114 °C. The melting point of pure melatonin is 116-118 °C. A H^1 NMR analysis was run on the synthesized melatonin and on stock melatonin (Appendix B: Figure 14). The spectrum of the synthesized melatonin is identical to the stock melatonin. The signal around 7-7.5 ppm is indicative of aromatic hydrogens. The peak around 2 ppm is the methyl group of the amide. The peak at 4 ppm corresponds to the second methyl group hydrogens attached to the oxygen off the aromatic ring. The peak at approximately 3 ppm corresponds to the hydrogens of the carbon attached to the amide.

Synthesized Melatonin vs. Pure Melatonin: Injection of Synthetic Melatonin and Collection of Hemolymph Samples

Synthetic melatonin and pure melatonin were injected simultaneously and hemolymph samples were taken from synthetic melatonin, pure melatonin and saline-injected crabs 3 hours post-injection. Synthetic melatonin and pure melatonin-injected crabs showed a higher lactate concentration after three hours when compared to saline injections (Figure 15, $P<.001$). The synthetic and pure melatonin-injected crabs displayed no significant difference in the lactate concentrations.

There was no significant difference between the synthetic melatonin, pure melatonin and saline-injected hemolymph glucose concentrations 3 hours post-injection (Appendix A: Figure 16).

DISCUSSION

The effects of melatonin on glucose and lactate concentrations were examined by injecting fiddler crabs with melatonin and collecting and assaying hemolymph samples. The hemolymph samples were compared to samples collected from seawater-injected and non-injected crabs. A comparison with non-injected crabs has never been done in previous research and provided some interesting results.

Melatonin caused a suppression of the glucose and lactate levels five hours following injection. However, the seawater-injected crabs had a more significant response to injection by demonstrating a larger decrease in hemolymph glucose and lactate concentrations. Injection seems to decrease circulating glucose and lactate concentrations in the hemolymph.

In order to determine why the seawater injected crabs showed significantly lower levels of glucose and lactate, hemolymph samples were assayed immediately after injection. The seawater-injected crabs had a significant rise in glucose levels while melatonin caused an initial suppression of lactate levels and glucose levels. The seawater injection initiated a stress response causing a rise in glucose concentrations. This indicates that the crabs are releasing large amounts of glucose in response to stress. Once released, the glucose is metabolized, as indicated by the downward trend in the amount of glucose. On the other hand, melatonin decreased the stress response by suppressing the

initial glucose concentrations. This is consistent with a study by Pierpaoli and Maestroni that reported melatonin's function as an anti-stress hormone.[24]

Hemolymph glucose and lactate levels follow a similar pattern showing a peak at approximately five hours after melatonin injection. Because they followed a similar pattern, lactate and glucose levels demonstrate a coupled response to melatonin. Because melatonin affects both lactate and glucose concentration in the hemolymph, it must affect the activity levels of the crabs and glucose metabolism.

Exogenous melatonin did not affect the timing of glucose and lactate peak because the glucose levels followed the same pattern as the non-injected crabs. However, melatonin had a significant effect on the amplitude of the peak by demonstrating a delayed hyperglycemic response. Melatonin administration causes the retention of glucose and lactate rhythmicity but decreases the release of glucose. The reduced glucose levels indicate that melatonin causes a decrease in the activity of the crabs. These results are consistent with the time-giving properties of melatonin because melatonin levels are normally highest during the night during periods of low activity. Melatonin injection caused the crabs to be less active and consume less glucose and produce less lactate as a biproduct.

The melatonin synthesis did in fact yield biologically active melatonin. None of the crabs died from the synthetic melatonin injection and the synthetic melatonin and pure melatonin-injections caused significantly similar responses in the hemolymph lactate concentrations. The effects on glucose concentrations were less conclusive because synthetic melatonin, pure melatonin and seawater injection induced the same response.

CONCLUSIONS AND FUTURE WORK

Melatonin has been shown to decrease the stress response to injection. However, it is not understood if the stress response is a result of the needle piercing the walking leg or the injection of fluid. By assaying the initial glucose and lactate levels with and without fluid injection, one can examine the cause of stress.

In addition, melatonin has been shown to maintain glucose rhythymicity while demonstrating a delayed hyperglycemic response. It is clear that melatonin induces a coupled response in glucose metabolism and lactate production, however the exact metabolic pathway has yet to be understood. One could investigate the effects of melatonin on insulin release as well as the glycolytic effects on tissues, such as the muscle and hepatopancreas. Melatonin has been shown to decrease in vitro insulin secretion from the rat pancreas.[25]

One could also investigate the effects of exogenous melatonin on the gluconeogenic pathway, glycogen breakdown and fatty acid oxidation. Melatonin could potentially be involved in one or more of these metabolic pathways to demonstrate a delayed hyperglycemic response.

Also, biologically active melatonin was successfully synthesized using the Fischer Indole synthesis. Because the yield was quite low, one could revise or attempt a new synthetic method to achieve a higher yield.

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Appendix A

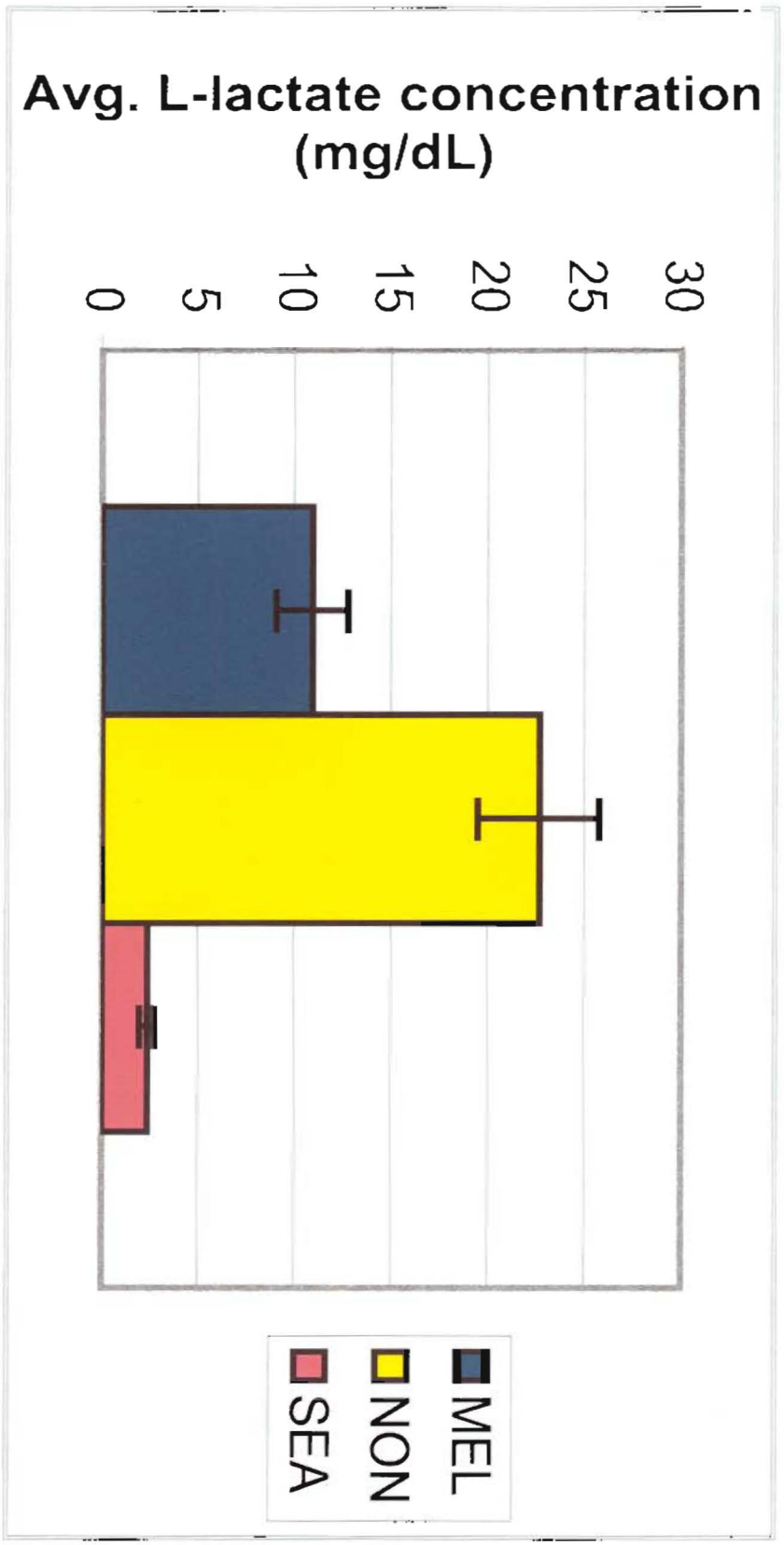


Figure 10: Hemolymph lactate levels in *U. pugilator* in response to 100 µL (50 µg/ml) melatonin and seawater injections at 1130h. Each bar represents the mean concentration of 25 crabs.

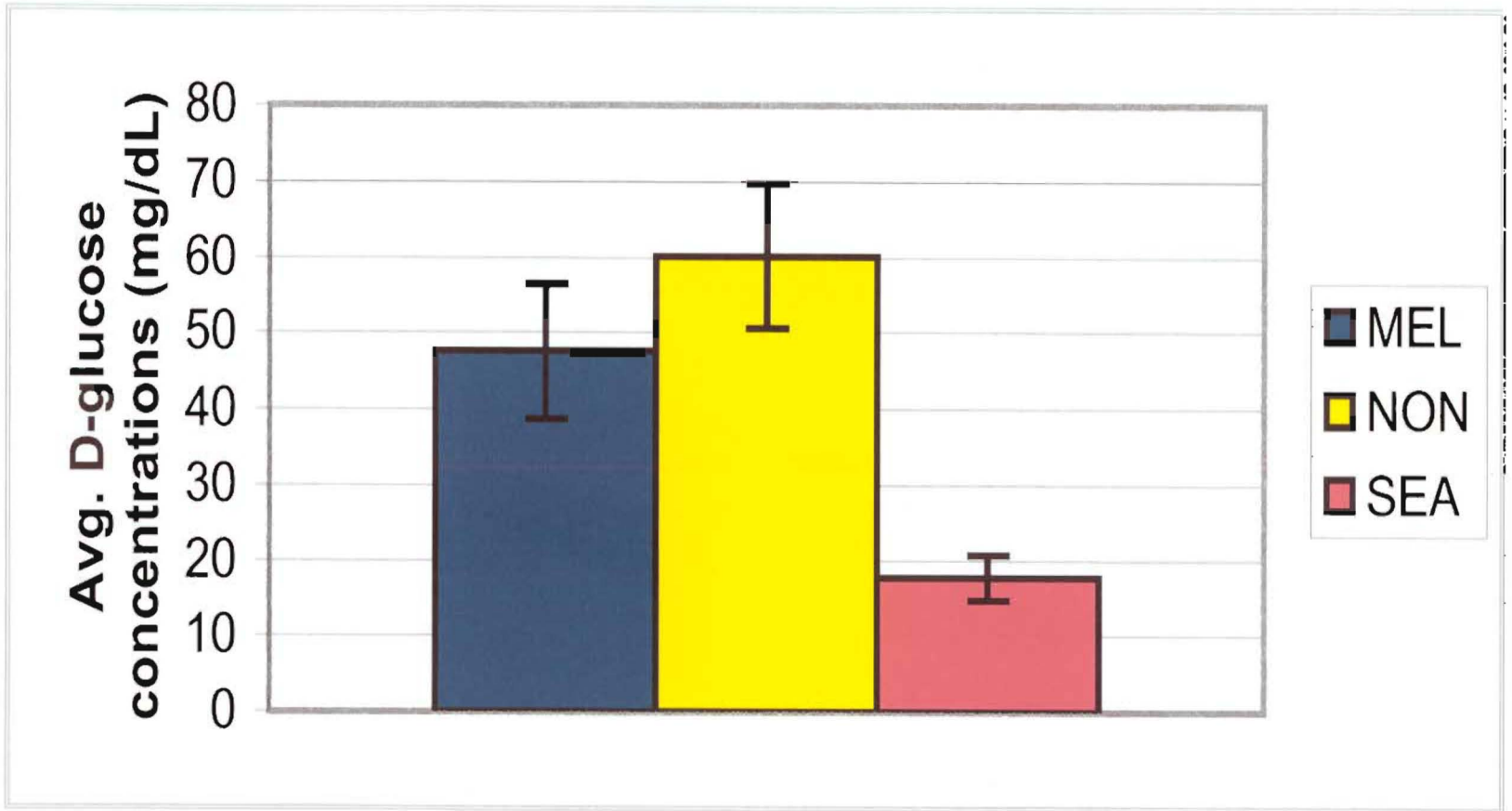


Figure 11: Hemolymph glucose levels in *U. pugilator* in response to 100 µL (50 µg/ml) melatonin and seawater injections at 1130h. Each bar represents the mean concentration of 25 crabs.

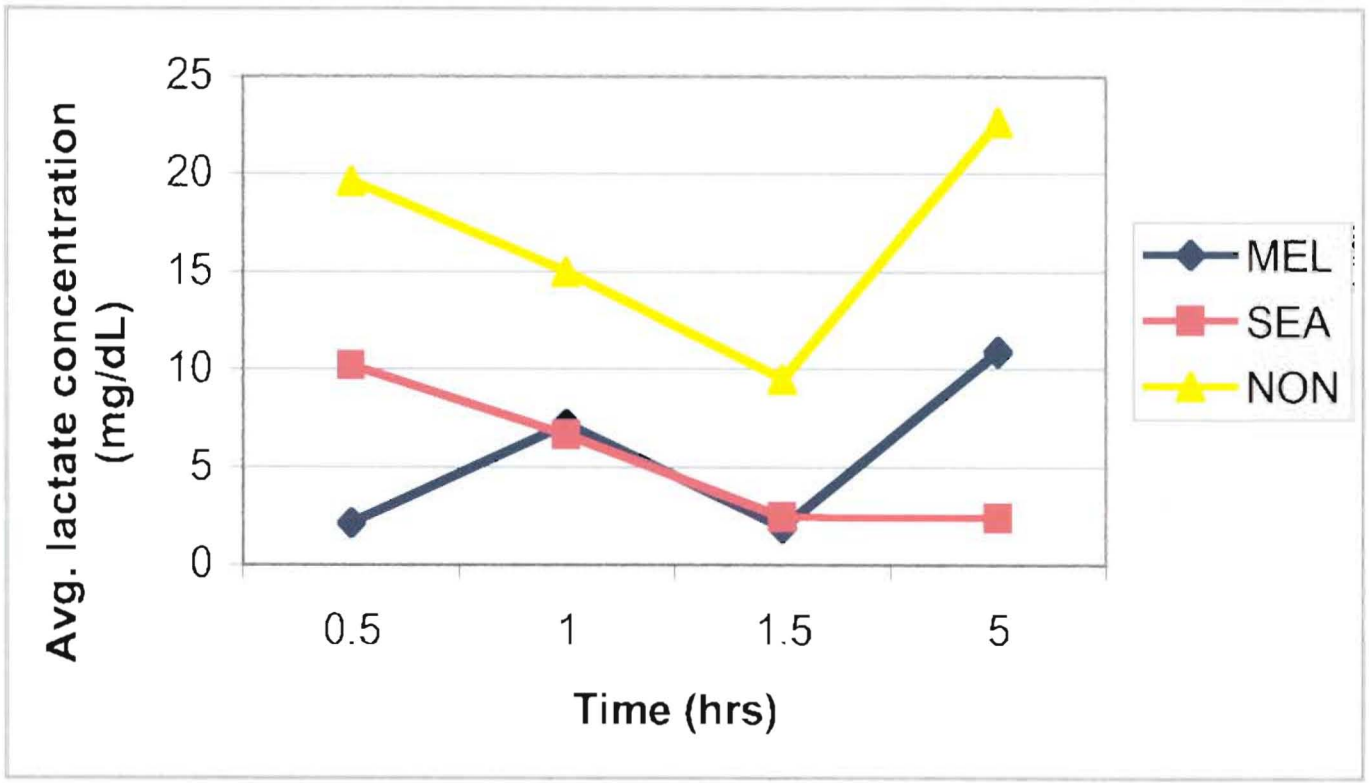


Figure 12: Average hemolymph lactate levels in *U. pugilator* in response to 100 µL (50 µg/ml) melatonin and seawater injections 0.5, 1.0 and 1.5 hours after injection compared with non-injected crabs. Each point represents the mean concentration of 15 crabs.

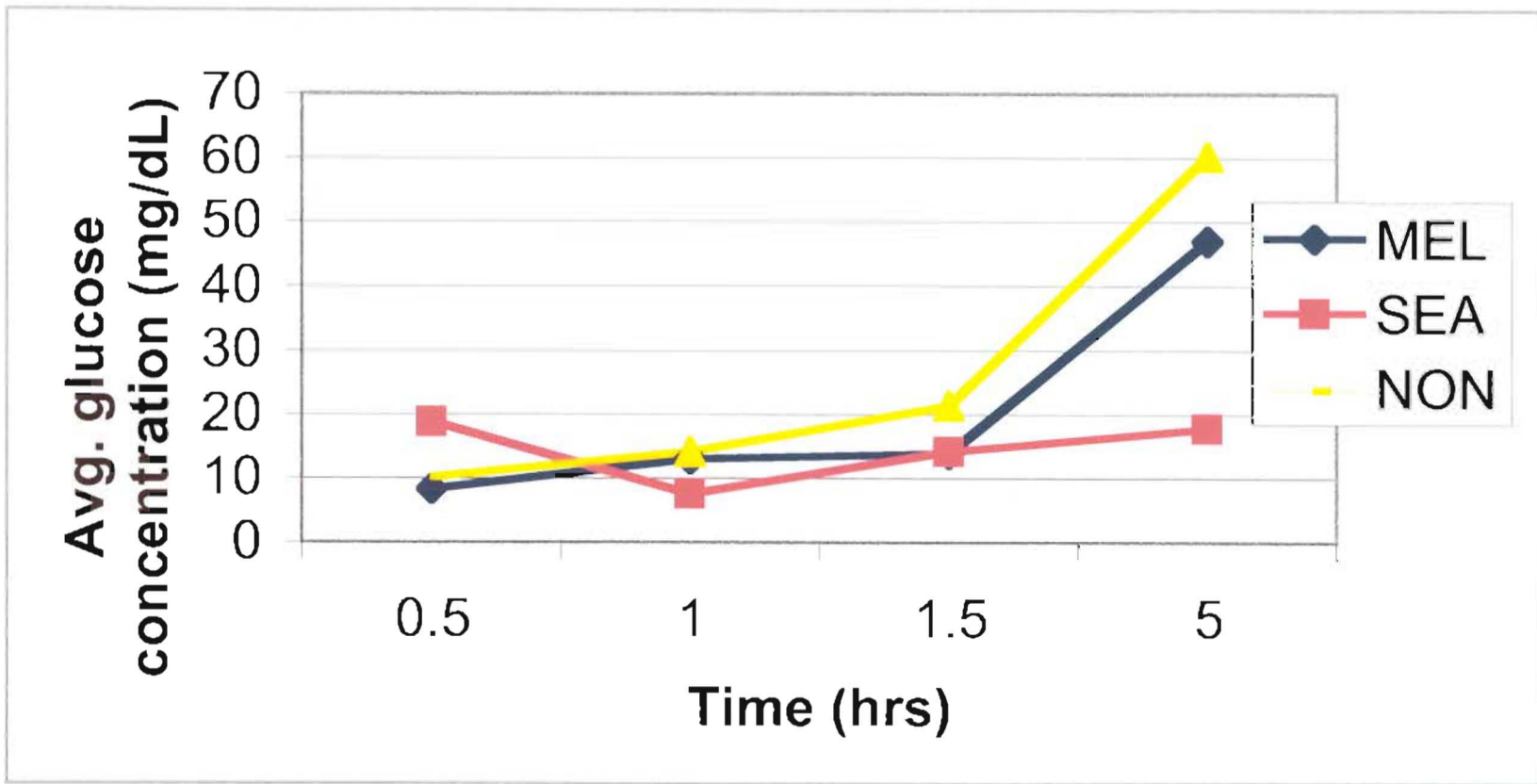


Figure 13: Average hemolymph glucose levels in *U. pugilator* in response to 100 µL (50 µg/ml) melatonin and seawater injections 0.5, 1.0 and 1.5 hours after injection compared with non-injected crabs. Each point represents the mean concentration of 15 crabs.

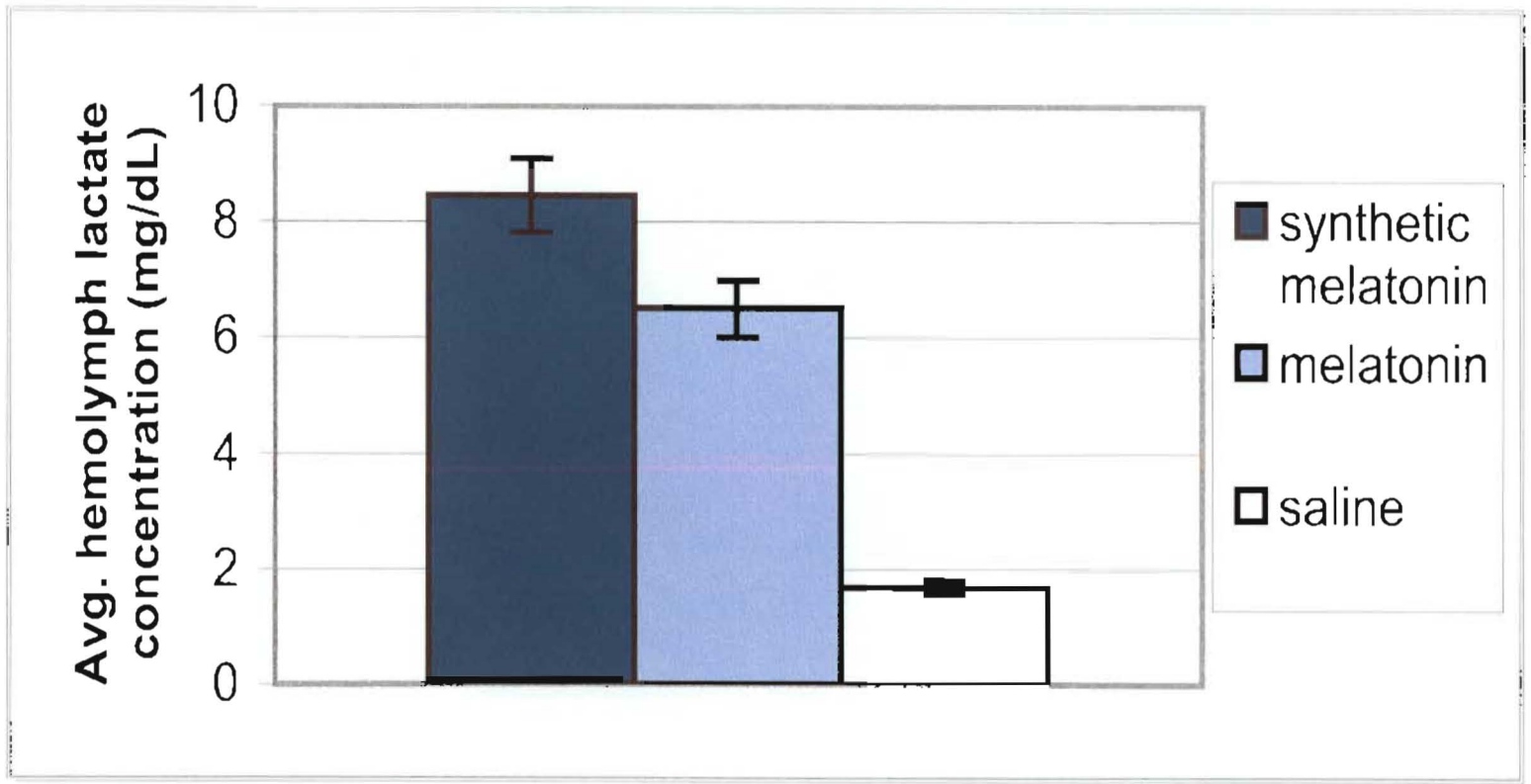


Figure 14: Average hemolymph lactate levels in *U.pugilator* in response to 100 L (50 g/ml) synthetic melatonin, pure melatonin and seawater injections 3 hours after injection. Each bar represents a mean concentration of 15 crabs

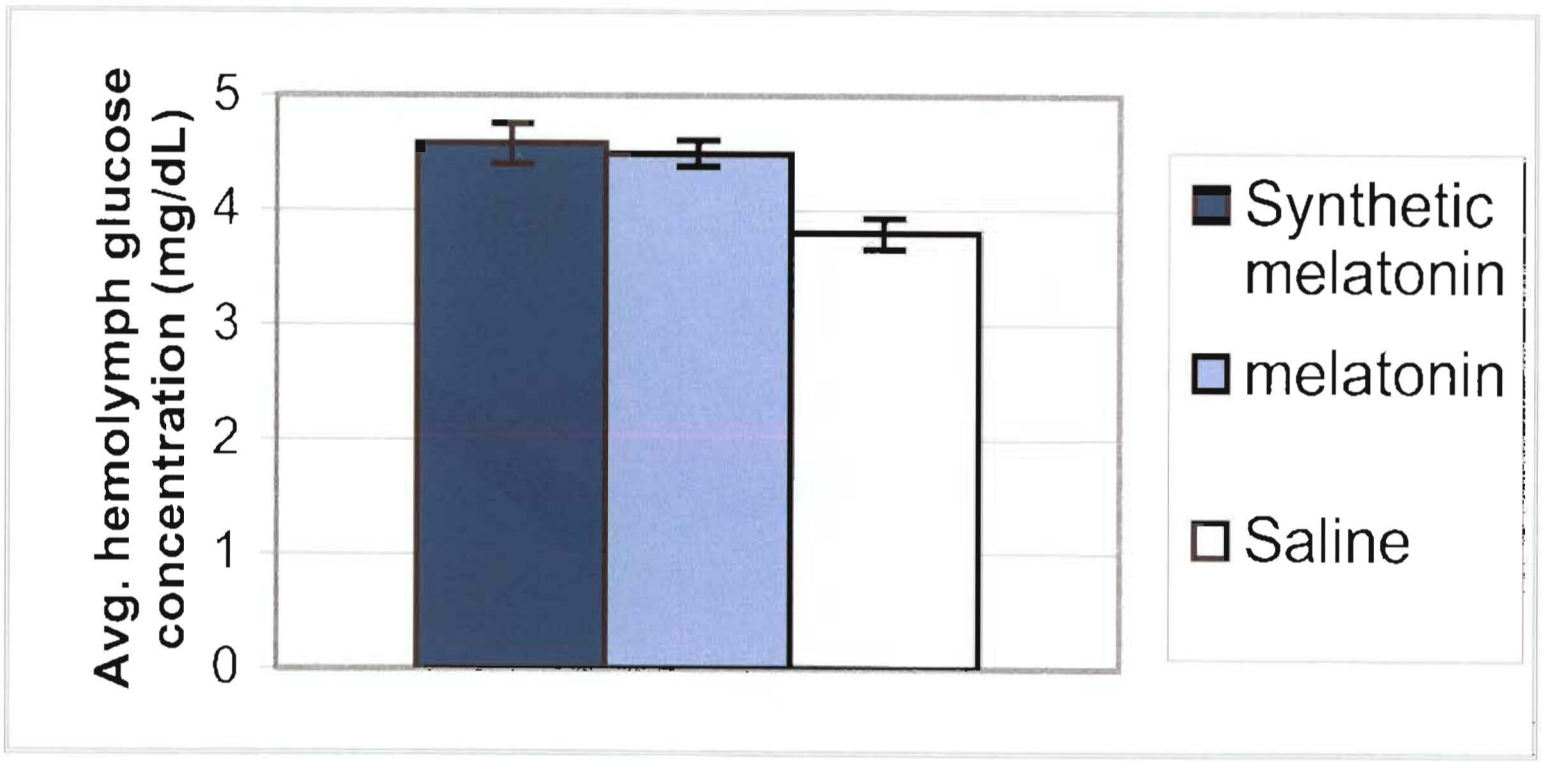
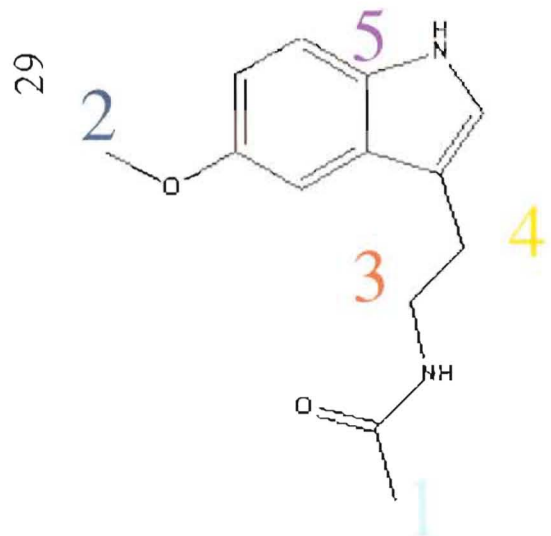


Figure 15: Average hemolymph glucose levels in *U. pugilator* in response to 100 µL (50 µg/ml) synthetic melatonin, pure melatonin and seawater injections 3 hours after injection. Each bar represents a mean concentration of 15 crabs

Appendix B: Figure 14:Melatonin: Spectral data

Synthetic Melatonin
Proton NMR

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