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Developmental steroids and vitamins: identified and quantified in predatory lady beetles

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Developmental Steroids and Vitamins; Identified and Quantified in Predatory Lady Beetles

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ABSTRACT

Autumn brings ladybeetles into homes to seek shelter during the winter, which is a nuisance. Their bites may induce an allergic reaction resulting in painful rashes. Predatory ladybeetles have been used as a biological control agents. Not only do they feed on ecological pests, they also feed on the saps of ripe fruits reducing the value of the damaged crop. Two species of ladybeetles (Coleoptera: Coccinellidae) will be examined to determine the quantities of steroids and vitamins used throughout their life stages. The species examined are native, *Hippodamia convergens*, of North America, and an invasive, *Harmonia axyridis*, of Asia. The species *H. axyridis* was collected throughout the Chattanooga, Tennessee, and *H. convergens* was purchased from California. It was of interest to determine the synthesis of the biomolecules as a mean to develop agents to control their overpopulation of the invasive species and increase the population of the native beetle. Each ladybeetles gender was determined and breeding pairs were placed in their own petri dish. They were incubated (22°C) and fed fava bean aphids. Each life stage of the larvae was collected, weighed, measured, and stored (-20°C). Digestions were conducted with a methanol-hexane mixture, and filtered to remove any excess biomass. Standards of biomolecules were prepared to determine their quantities within the samples. The investigation was able to identify at micron concentrations the steroids and vitamins produced by these two species. The results showed that *H. convergens* had a 2-fold more estradiol than *H. axyrids* though they were subjected to identical breeding conditions. Between the second and third instar, there was a 6-fold increase of estradiol in *H. axyridys*. The beetle *H. convergens* had a 4-fold increase in estradiol production. Cholesterol and vitamins were also identified within the digested samples indicating that these two species produce these biomolecules for development.

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INTRODUCTION

Vitamins and steroids play a key role in the proper development of organisms. When a developmental steroid or vitamin is affected by external stimulus, it could hinder or escalate their growth rate. Predatory lady beetles use these molecules to develop effectively and efficiently. Lady beetles, especially non-native species, are beginning to overpopulate and becoming a nuisance in the United States. By looking at several vitamins and steroids as control agents, their population could be managed by altering their metabolic pathways. To target their growth, it was important to determine the concentrations of these molecules at various life stages of the two species, a native, *Hippodamia convergens*, and an invasive, *Harmonia axyridis* (Figure 1) (Camarano et al, 2006; Evans et al, 2011; Kajita et al, 2015; Majerus et al, 2006). Since few studies have been conducted to identify vitamins and steroids, the purpose of this research is to determine the quantities of these biomolecules to find a way to effectively manage the populations without the use of harmful pesticides.

The taxonomy of lady beetles begins in the domain of Eukarya then branch into the Animalia kingdom (Hodeck and Honek, 2011). Notably, lady beetles are assigned to the largest phylum, Arthropoda, and order, Coleoptera. These insects reside in the family Coccinellidae, which is exceptionally large as well. The lady beetles that were used in this work were *Harmonia axyridis* and *Hippodamia convergens*. The genus refers to the type of lady beetle, *Harmonia,* whereas the species indicates the individual lady beetle, *axyridis*.

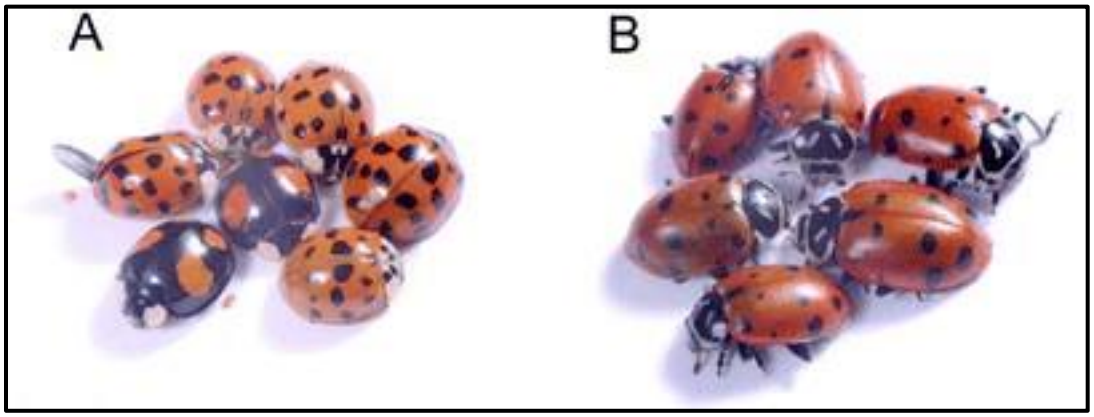


Figure 1: Adult lady beetles

A) The invasive species *Harmonia axyridis* are also called multicolored Asian lady beetles due to the multiple colors their elytra can come in. B) The native species *Hippodamia convergens* are more oval in shape and their elytra is a rust orange color.

They have been used for ecological control of aphids, coccids, and psyllids for many years (Camarano et al, 2006; Evans et al, 2011; Kajita et al, 2015; Majerus et al, 2006). Unsuccessfully, *Harmonia axyridis* were first introduced in America in 1916, to control aphids and scale insects populations (Camarano et al, 2006; Evans et al, 2011; Kajita et al, 2015; Majerus et al, 2006). They are native to central and eastern Asia, and were chosen for their size, diverse directory range, efficiency, and niche colonization ability (Camarano et al, 2006; Daloze et al, 1995; Majerus et al, 2006). They failed to establish effective colonies until 1988, when they were discovered in Louisiana (Evans et al, 2011; Majerus et al, 2006). Since then, they have spread across most of the United States and Canada, and are considered the most common ladybeetle in North America (Evans et al, 2011; Majerus et al, 2006). After the initial discovery of a colony in Georgia, *H. axyridys* spread through the state rapidly; and within two years, they have covered approximately 149,977 km^2 (Majerus et al, 2006). The success of their colonization is partly due to their multivoltine ability in North America since food and climate are ideal for breeding throughout the spring, summer, and fall (Daloze et al, 2005; Evans et al, 2011; Lombaert et al, 2014; Majerus et al, 2006). Their great phenotypic plasticity makes them competitors in a wide variety of ecosystems and allows them to colonize rapidly (Camarano et al, 2006; Lombaert et al, 2014; Majerus et al, 2006). These attributes contribute to *H. axyridis* use as a control agent, but there are some negative effects to the use of species *axyridis*. They have many different developmental steroids and vitamins that allow them to develop and reproduce efficiently as well as effectively. There reproduction system has allowed them to overpopulate and damage many crops that they were used as a control agent (Evans et al, 2011).

A mutation in some of the lady beetles was discovered rendering them flightless (Cripps et al, 1994; Ferran et al, 2000; Tourniaire et al, 2000). In an in-depth study of these flightless beetles, it was noted that there was a mutation in the gene that produces myosin thus altering the formation of its wings. The lady beetles with this mutation were collected and bred in order to form a colony of flightless off-springs. These beetles were unable to move long distances thus controlling aphid populations in one area more efficiently (Cripps et al, 1994; Ferran et al, 2000, Tourniaire et al, 2000). This finding helped to control the invasion of lady beetles since they were unable to migrate outside of their release site.

Kajita (2015) identified some defensive compounds in several species of ladybeetles, including *H. axyridis*. Lady beetles are intra-guild predation thus feeding on eggs and larva. Harmonine is an alkaloid defensive compound produced by many lady beetles, but *H. axyridis* was found to produce higher concentrations in their vulnerable early stages of life. Many other species had a high mortality rate when they ate the eggs and larva of *H. axyridis* since they could not metabolize the defensive compound. Studies have shown that *H. axyridis* did not show signs of delayed development or high mortality rates when fed a diet of their own eggs (Kajita et al, 2015; Kajita et al 2014).

Developmental steroids play a key role not only in the maturation of the lady beetle larvae but the synthesis of various proteins. The protein vitellogenin (Vg) is a lipid transfer protein that is responsible for the production of the egg yolk as a nutrient

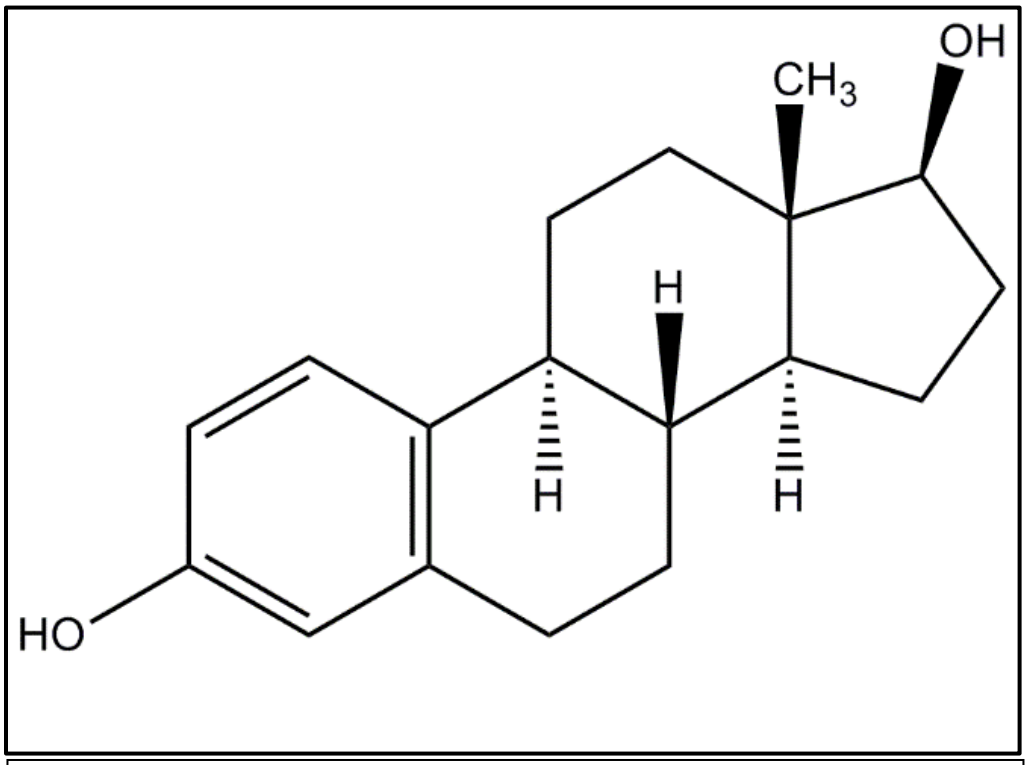


Figure 2: Estradiol

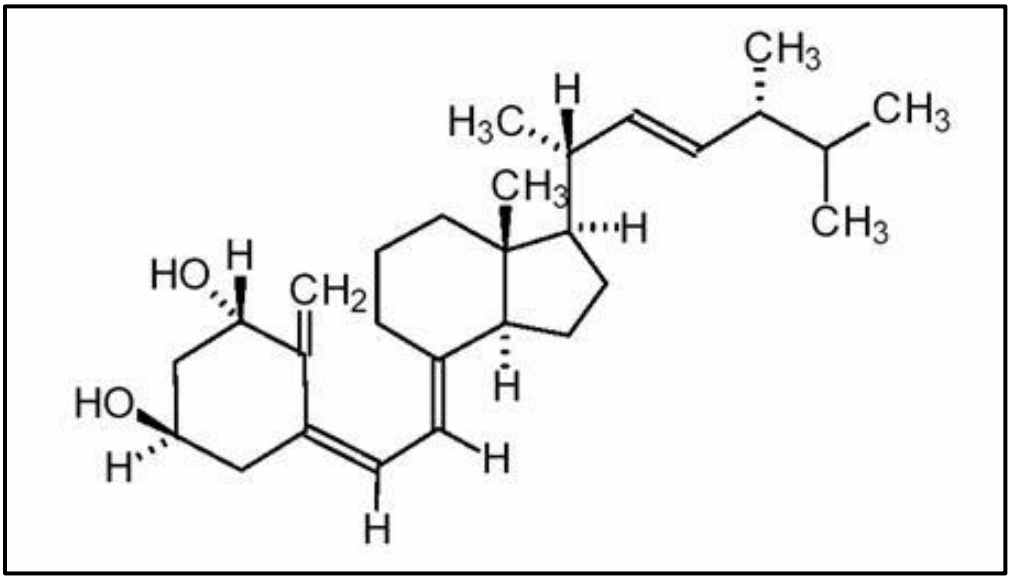


Figure 3: Vitamin D2 (ergocalciferol)

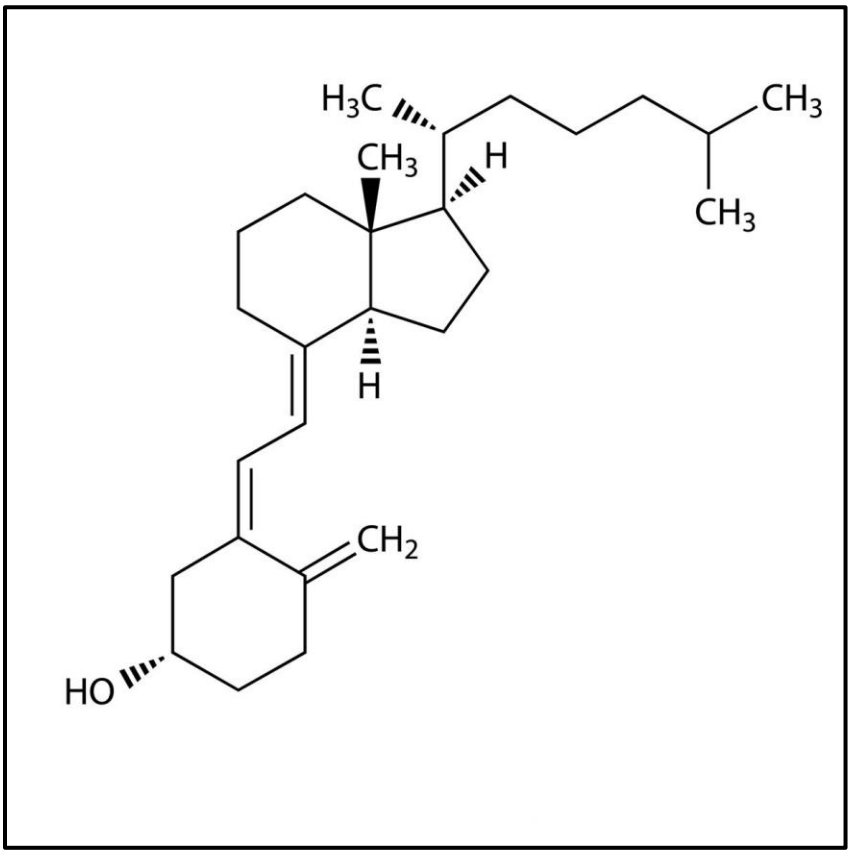


Figure 4: Vitamin D3 (Cholecalciferol)

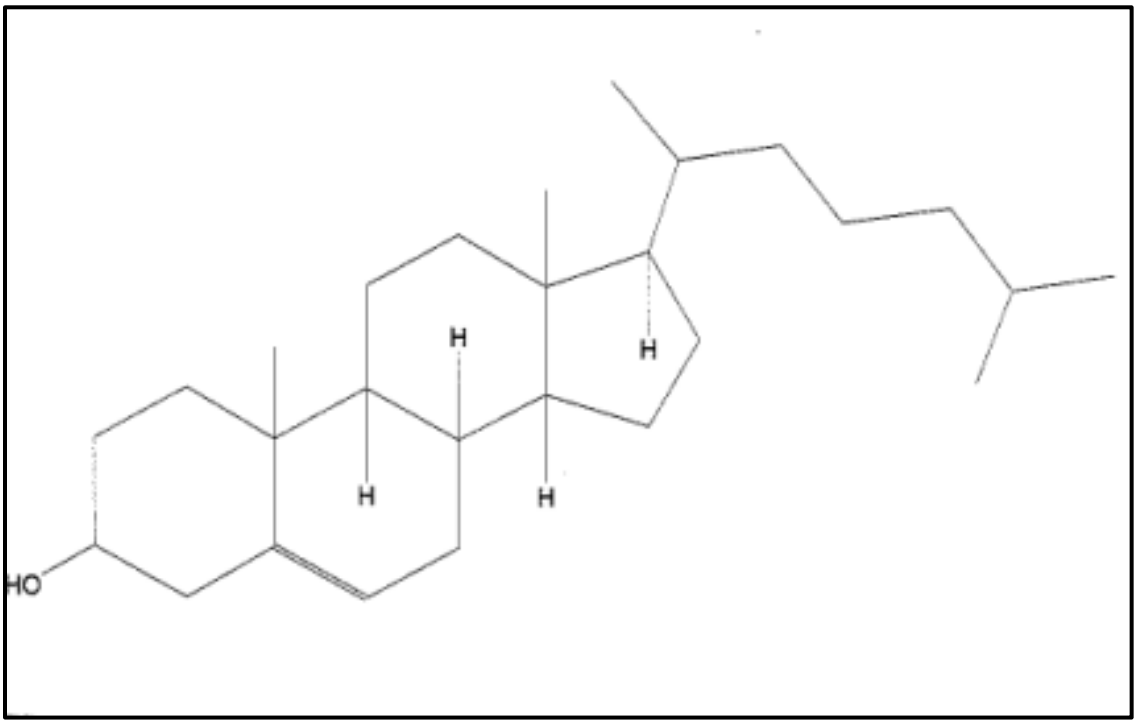


Figure 5: Cholesterol

source for developing embryos and is a steroid induced protein (Tufail et al, 2014; Wiley et al, 1981). It is synthesized in the fat body and secreted into the hemolymphatic system, which allows it to carry nutrients to the ovaries. The expression of the Vg gene is under hormonal control mainly by juvenile hormone and estrogens. The juvenile hormone regulates the Vg production in ladybeetles and can also prevent molting which is carried out by ecdysone (Chapin and Brou, 1991; Gordon et al, 1985; Meinwald et al, 1985; Schildknecht, 1970; Tufail et al, 2014). It was also found that exposing ladybeetles to cadmium delayed ovary maturation which would delay reproduction (Tufail et al, 2014). The structure of the molting hormone ecdysone is relatively similar to cholesterol and vitamin D. There could be a metabolic pathway that could be signaled by lower or higher concentrations of these hormones to synthesize each other.

The invasive species, *Harmonia Axyridis*, and native species *Hippodamia Convergens,* was studied through all four of their instar stages along with the adult stage. As the lady beetles progressed through their larval stages, an increase in each of the developmental chemical is expected to be observed. Ultraviolet-visible spectroscopy, fluorimeter, high performance liquid chromatography, and gas chromatography-mass spectroscopy were used to identify, analyze, and quantify the different steroids and vitamin molecules that they produce. Potential developmental steroids and vitamin compounds that will be specifically identified and quantified will be estradiol (Figure 2), Vitamin D2 (Figure 3), Vitamin D3 (Figure 4), and cholesterol (Figure 5).

By investigating developmental biomolecules in these species, stages could be identified where a control agent could be introduced to alter the development of the invasive and native beetles. The concentrations of estradiol could be used as an

identification for the gender of the instars of the ladybeetles, since gender between larva is undistinguishable. The early identification of the gender could provide other methods of population control.

MATERIALS AND METHODS

The standards prepared for the developmental steroids and vitamins, estradiol, vitamin D_2, vitamin D_3, and cholesterol, were purchased from Sigma-Aldrich. The solvents, hexane, methanol, formic acid, and HPLC grade water, were all purchased through Fisher.

The preparation of the instars for digestion followed a modified protocol using the filtration apparatus (Figure 6) (Perry and Santiago, 2015). *Harmonia axyridis* adults were collected from the surrounding Chattanooga area in October 2015 (Figure 7). A second species of adult lady beetle, *Hippodamia convergens*, were purchased from Arbico Organics Company (www.arbico-organics.com/) in May 2015. Each species of lady beetles was divided into breeding pairs and placed into individual petri dishes that were 9 cm in diameter and 1.5 cm in height (Kajita et al, 2015). Each pair was provided a folded piece of paper to lay their eggs on along with a piece of cotton saturated in water to keep them hydrated. The breeding pairs and the larvae were kept in an incubator set at approximately 22°C. Both species of lady beetles were provided a diet of pea aphids (*Acyrthosiphon pisum*) that were raised on fava beans (*Vicia faba L.*). First to fourth instar larvae were collected within 24 hours after molting. Each instar was then transferred into a 1.5 mL snap cap tube and stored at -20°C in a freezer. They were later weighed and measured.

Figure 6: filtration apparatus

A ring stand was used along with clamps to hold all the pieces of the filtration apparatus in line with each other

Figure 7: map of Chattanooga and surrounding areas.

The boxes highlighted in red show the areas in which the *Harmonia axyridis* ladybeetles were collected from. Site 1 is the campus of the University of Chattanooga, and site 2 is on Boss rd in Chickamauga Georgia.

In previous studies of Coccinellid eggs, a protocol for their digestion was modified for use with the instars (Camarano et al, 2006; Kajita et al, 2015; Perry and Santiago, 2015). For the first through second instars, five larvae were digested. For third or fourth instar, one larva were digested. All larvae were digested in 200 µL of methanol with an addition of 400 µL of hexanes. The contents of the digestion was filtered through a column containing glass wool. The column was rinsed twice with 120 µL of methanol. The glass wool column filtrate was then filtered through a syringe filter to remove any excess biomass (Figure 6). Since hexane and methanol have different densities with hexane being lighter than methanol, the mixture was separated into different capped vials. The hexane layer was removed and placed in a snap cap labeled sample C. The hexane collection was evaporated and sample was resuspended in 400 µL of methanol. The methanol layer was equally split between two snap cap vials and brought up to a volume of 800 µL each, then labeled sample A and B.

Standards were prepared with each of the vitamins and steroids that were analyzed, namely estradiol, vitamin D_2, vitamin D_3, and cholesterol. Standards were prepared in methanol with the concentrations consisting of 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 21.875 µg/mL, and 15.625 µg/mL. Initially, the highest concentrations were analyzed on the UV-VIS to initially determine the maximum wavelengths. To confirm the wavelength, samples were analyzed in the fluorimeter where the wavelength found on the UV-VIS as a comparison. Emission and excitation spectra were used to identify the analytes on the Agilent 1220 Integrity HPLC.

As a means to determine the appropriate column, the polarity of the steroids and vitamins were reviewed prior to analyzing the molecule with the liquid chromatograph.

The column used for estradiol and cholesterol was an Agilent reverse phase Proshell 120. The column used for vitamin D was an Agilent Zorbax Eclipse plus C-18. Instrument protocols were developed relative to the same molecular characteristics. The mobile phase to identify estradiol contain methanol and 0.1% formic acid. A gradient was chosen over an isocratic ratio of the mobile phase to allow for the separation and elution of molecules. The flow rate of 1.00 mL per minute was used throughout the 10.00 minutes experiment with a five-minute post run. The post run allowed time for the instrument to equilibrate to the original buffer ratios and elute any residue molecules remaining on the column. The gradient solvent phase started with 25% formic acid and 75% methanol, and continued until the percentage formic acid reached 2.5% formic acid. The protocol for Vitamin D was created using a mobile phase of methanol and acetonitrile (Turpeinen et al, 2003). This method used a gradient similar to the protocol used to identify estradiol with a run time of 13.00 minutes. Varying concentrations of vitamins or steroids were used to produce a standard curve. For vitamin D, it was expected that a mixture of vitamin D_2 and vitamin D_3 in the sample would have similar retention times, because of their structure similarities. A solution was made with similar vitamin concentrations that will be used as the standard marker to identify the retention time indicating the presence of the vitamin. Cholesterol used a gradient mobile phase of acetonitrile and methanol. This protocol solution mixture initially started with 85% acetonitrile to 15% methanol until the percentage of methanol was 0%, which was held for 4.00 minutes with a total run time of 10.00 minutes.

All larval stages for three breeding pairs of each species were digested using the digestion protocol (Camarano et al, 2006; Kajita et al, 2015; Perry and Santiago, 2015). A

digestion of a second set of larvae was performed using the same breeding pairs to compare not only the species but siblings. Each sample was them used to determine concentrations of the vitamins or steroids during this study. The first breeding pair offspring of *H. axyridis* and *H. convergens* was analyzed on the liquid chromatograph. A volume of standard addition was used to determine the low concentration of the analyte. Equal amounts of the standard and the sample were placed in a vial for analysis. The minimum volume (250 mL) was used to run the sample twice due to the instruments volume extraction limit (50 µL). Experiments were conducted twice to derive a standard deviation.

The peaks that corresponded to the vitamins or steroids being investigated on the HPLC were collected, and then injected into the GC-MS to identify the molecule. The molecular weight of estradiol is 272.28 g/mol and the melting point is 151-152°C. The protocol used had an initial temperature of 60.0°C and held for 3.00 minutes. It was then ramped up at 30°C/min to a temperature of 270°C and held for one minute. The final phase of the analysis increased the temperature by 10°C/min to 325.0°C and held for two minutes. The final holding temperature was set to ensure residue molecules would be eliminated from the column. A sample volume of 6 µL where 95% was used to identify estradiol in the samples due to low analyte concentration levels. The protocol for vitamin D had an initial temperature set at 40°C and held for two minutes then raised 150°C at a rate of 10°C/min and held for two minutes. The last ramp had a rate of 30°C/min until a temperature of 325°C was reached and held for two minutes to eliminate residual compounds remaining in the column from the sample. The protocols of the GC-MS were

created by taking into consideration the molar mass of the molecules along with the melting point to allow for sensitivity and separation.

RESULTS

For the organisms to increase in size and weight, varying concentrations of developmental steroids or vitamins will be necessary throughout their life stages. A comparison of weight and length of each species during development can indicate varying concentrations of these compounds. Measurements of all the instars were determined after collection this includes the length and the weights at each larval stage. Standard deviations were also calculated for each species at each stage of development to look at differences in growth between species. Weights were recorded in micrograms and the lengths in millimeters. Tables 1 thru 5 show the averages of the weights and lengths of all instar levels and adults along with their standard deviations. Comparisons between species found that they have similar lengths until they reach adulthood. In their adult stage of life, a difference of 0.6 mm is observed between the species which was the greatest observed difference in length between species. The initial observed increase in weight between the species was during the third instar stage of development with a 2-fold increase of *H. axyridis* over *H. convergens*. In the overall development of the species, *H. axyridis* increased by a fold of 183, where *H. convergens* had a 108-fold increase in body weight.

Estradiol, cholesterol, vitamin D_2, and vitamin D_3 standards were all identified with the use of several instruments. UV-VIS was used first to determine the excitation

Table 1: 1st instar weights in micrograms and measurements in millimeters

1st instar				
Species	Mean µg	RSD	Mean mm	RSD
Hippodamia convergens	195	0.224	1.1	0.09
Harmonia axyridis	197	0.279	1.1	0.16

The eggs from the breeding pairs were placed in a petri-dish and kept in an incubator until they hatched. The 1st instars were then collected in two sets of ten from each breeding pair and were placed in a freezer. Weights and measurements were taken of each instar and the averages were recorded along with a standard deviation.

Table 2: 2nd instar weights in micrograms and measurements in millimeters

2nd instar				
Species	Mean µg	RSD	Mean mm	RSD
Hippodamia convergens	783	0.123	2.4	0.17
Harmonia axyridis	751	0.221	2.5	0.06

The 1st instars were fed a diet of fava bean aphids and kept in an incubator to develop. After the 1st instars molted into 2nd instars they were collected into two groups of ten from each breeding pair and placed in a freezer. Weights and measurements of each instar were taken and the averages were recorded along with the standard deviations.

Table 3: 3rd instar weights in micrograms and measurements in millimeters

3rd instar				
Species	Mean µg	RSD	Mean mm	RSD
Hippodamia convergens	2417	0.082	4.0	0.06
Harmonia axyridis	3495	0.165	4.1	0.10

The 2nd instars were fed a diet of fava bean aphids and kept in an incubator to develop. After the 2nd instars molted into 3rd instars five were collected from each breeding pair and placed in a freezer. Weights and measurements of each instar were taken and the averages were recorded along with the standard deviations.

Table 4: 4th instar weights in micrograms and measurements in millimeters

4th instar				
Species	Mean µg	RSD	Mean mm	RSD
Hippodamia convergens	11299	0.136	6.7	0.11
Harmonia axyridis	15291	0.182	7.0	0.14

The 3rd instars were fed a diet of fava bean aphids and kept in an incubator to develop. After the 3rd instars molted into 4th instars five were collected from each breeding pair and placed in a freezer. Weights and measurements of each instar were taken and the averages were recorded along with the standard deviations.

Table 5: Adult weights in micrograms and measurements in millimeters

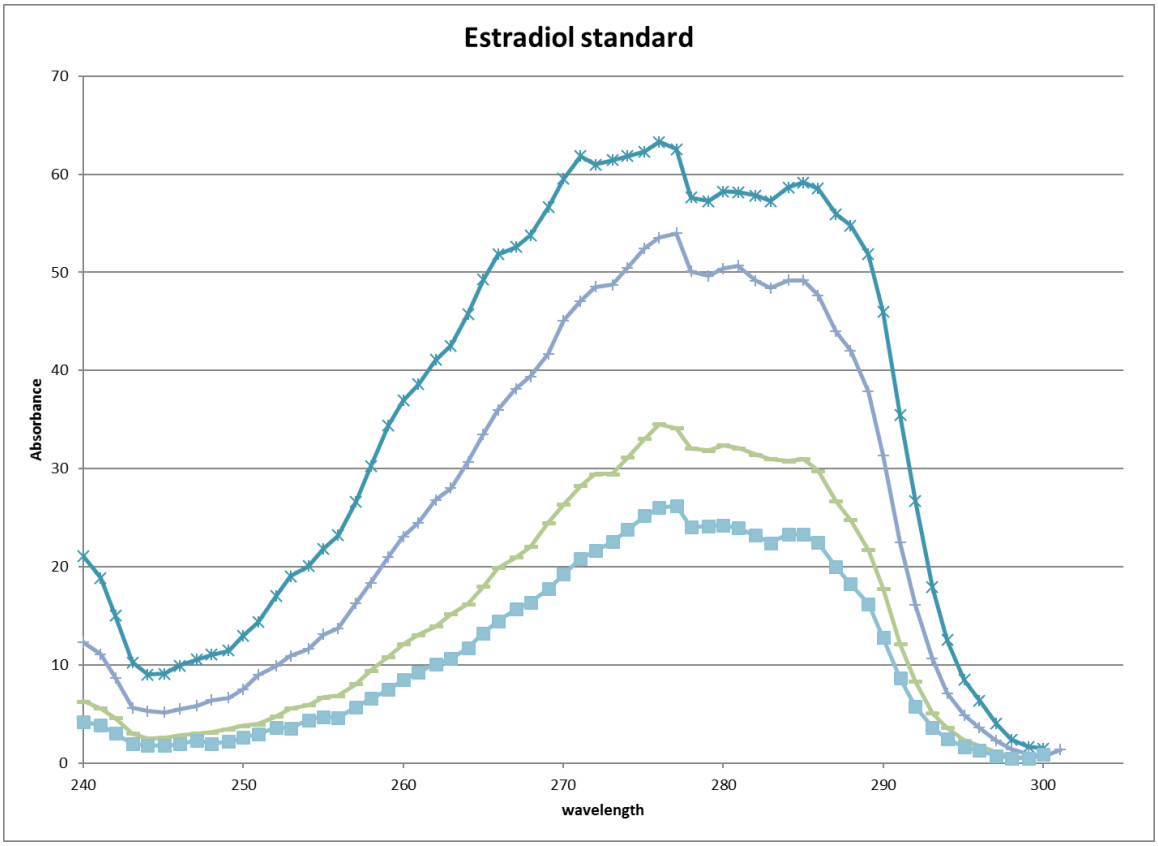
Adult				
Species	Mean µg	RSD	Mean mm	RSD
Hippodamia convergens	20948	0.196	6.8	-
Harmonia axyridis	35960	0.147	7.4	0.07

The adults used for breeding were kept in their own petri dishes in pairs. They were fed a diet of fava bean aphids and stored in an incubator. After all instar stages were collected the breeding pair adults were placed in snap-cap vials and stored in a freezer. Weights and measurements of each adult were taken and recorded along with the standard deviations

wavelength to be used in the fluorimeter for estradiol this was determined to be 270 nm, for vitamin D_2 was found to be 531 nm and 265 nm. A peak was found in the visible range for vitamin D_2 and was further investigated. Vitamin D_3 had a peak at 266 nm determined from the UV-VIS.

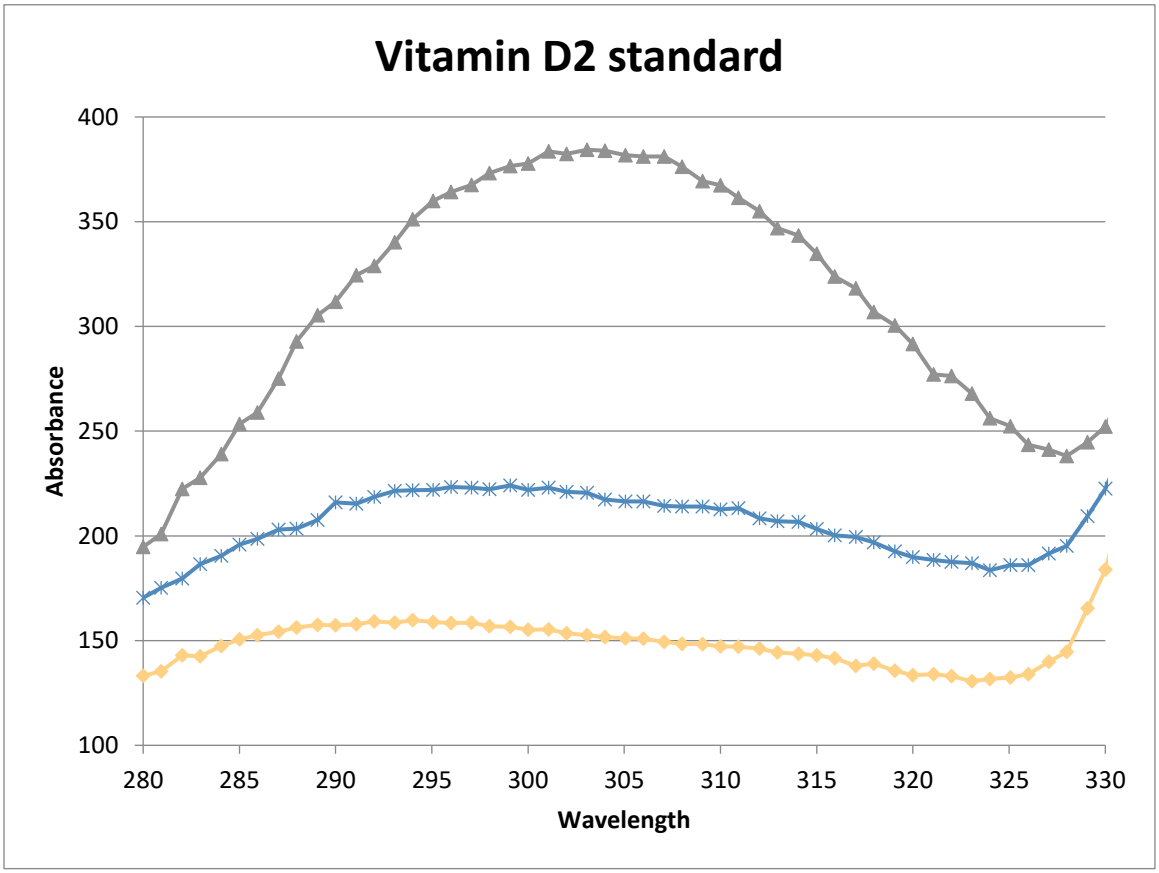
The wavelengths of the serial dilutions of the estradiol standards were observed on the fluorimeter. Graph 1 shows the chromatogram from the fluorimeter of estradiol standards. This graph indicates the decreasing intensity as the concentration of estradiol decreases. The lambda max (λ_{max}) for all the concentrations were 280 nm. Graph 2 shows the fluorimeter results for vitamin D_2 with three decreasing concentrations. The λ_{max} for all three concentrations was found to be 304 nm. The intensities of the samples decreased with the decrease in concentration of vitamin D_2. The fluorimeter results for the standardization of vitamin D_3 is shown in Graph 3. There were two λ_{max} values found. The λ_{max} values for the three concentrations were 219 nm and 293 nm respectively. These wavelengths were used when developing the HPLC protocol that would aid in the further analysis of these biomolecules.

The λ_{max} that was determined from the fluorimeter for the standard of the vitamins and steroids were used on the high-pressure liquid chromatography (HPLC) instrument to calculate a standard curve. The integrated area of the peaks of each concentration of estradiol was used to determine a linear regression. Graph 4 shows the curve along with



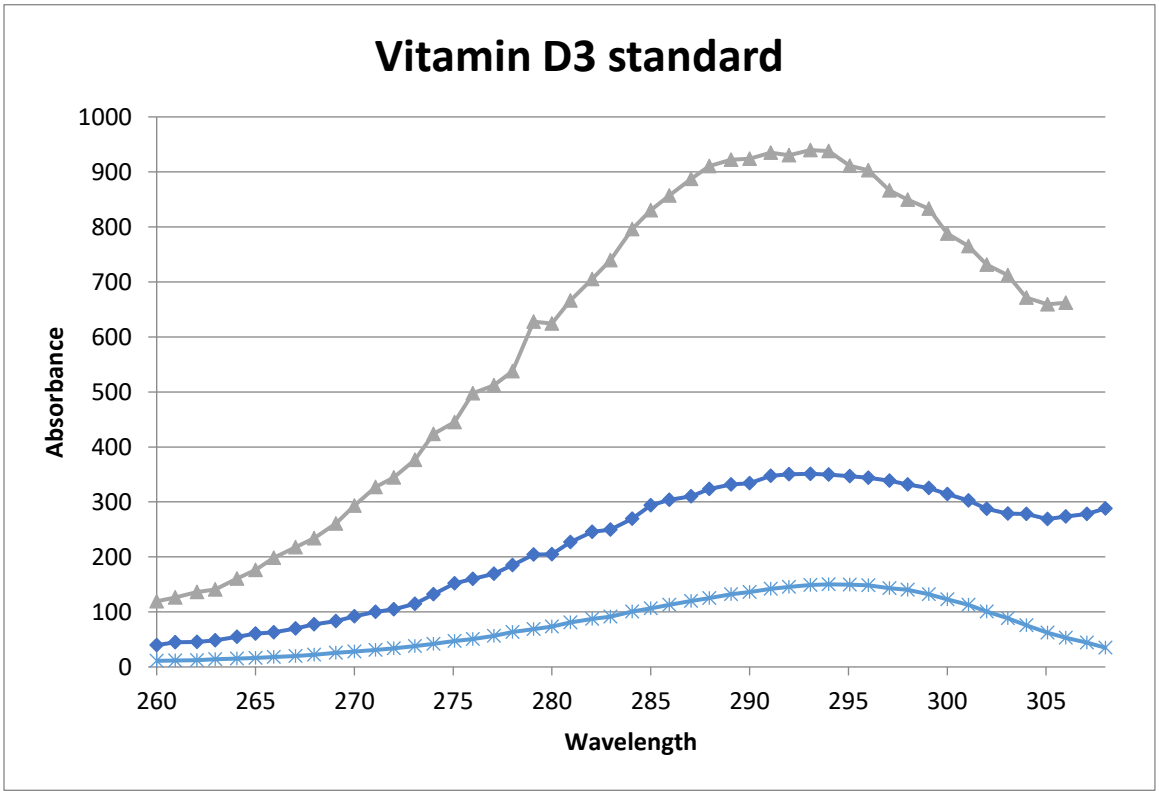
Graph 1: Fluorimeter results for estradiol standards.

The fluorimeter results for estradiol serial dilutions shows a decrease in absorbance as the concentration decreases. Concentrations ranging from 7.81 µg/mL to 125 µg/mL of estradiol in methanol.



Graph 2: Fluorimeter results of Vitamin D_2 standard

The fluorimeter results for vitamin D_2 serial dilutions shows a decrease in absorbance as the concentration decreases. Concentrations ranging from 7.81 µg/mL to 125 µg/mL of vitamin D_2 in methanol.

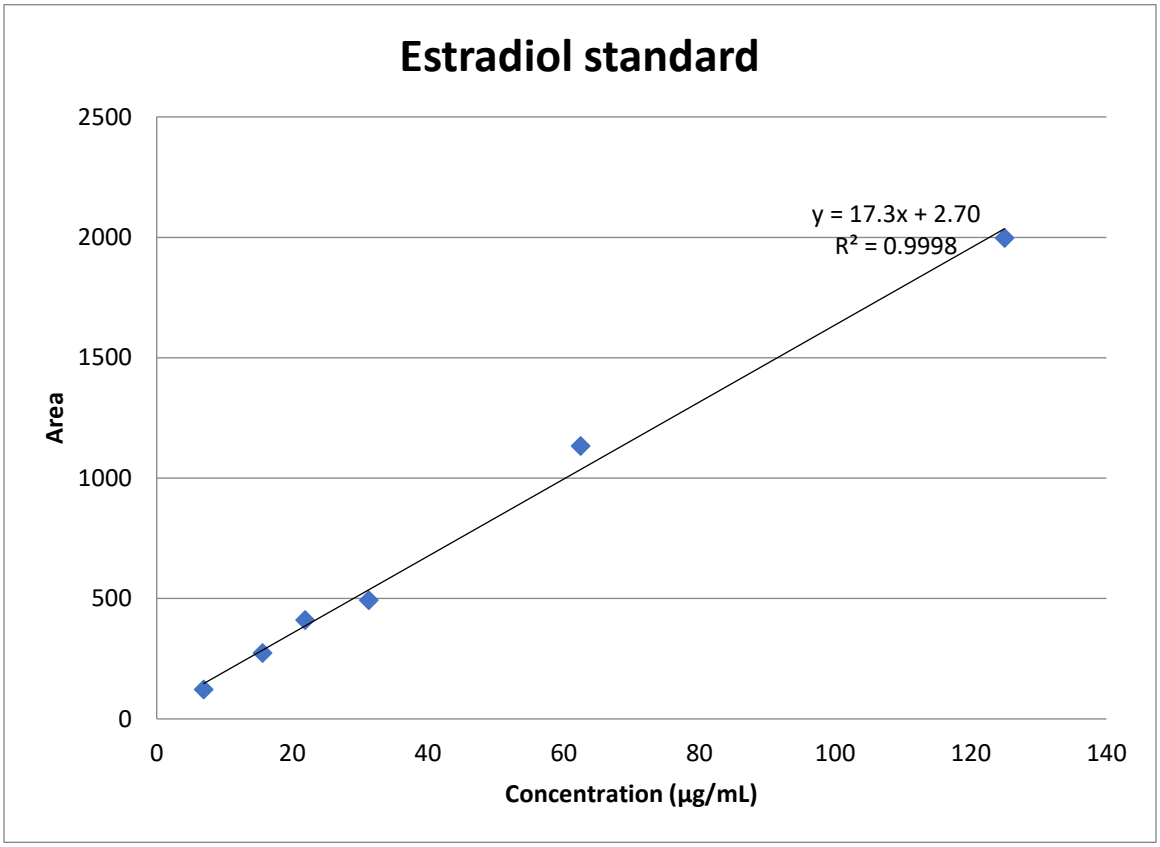


Graph 3: Fluorimeter results of Vitamin D_3 standard

The fluorimeter results for vitamin D_3 serial dilutions shows a decrease in absorbance as the concentration decreases. Concentrations ranging from 7.81 µg/mL to 125 µg/mL of vitamin D_3 in methanol.

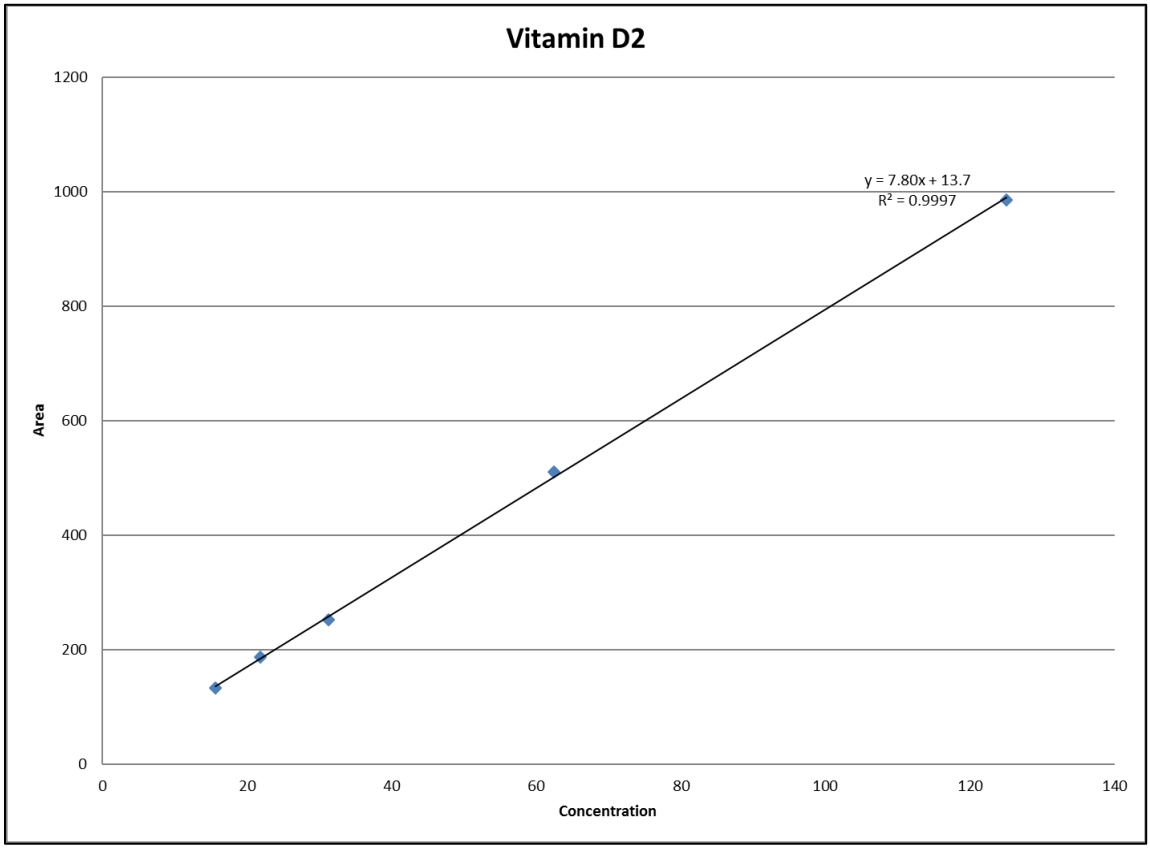
the equation of the line, y = 17.3x + 2.70, and the R^2 value of 0.9998, indicating the data correlates. By use of the HPLC, the standard curve in Graph 5 was produced. The λ_{max} determined from the fluorimeter was used in the protocol with the HPLC in order to determine the peak area of the different concentrations of vitamin D_2 to create the standard curve. This calculated equation (y = 7.80x + 13.7) has a correlation of 0.9997. The standard curve for vitamin D_3 has a R^2 value of 0.998, and an equation of y = 15.1x + 16.6, as shown in Graph 6. Since Vitamin D_2 and D_3 had similar retention times it was decided to run a serial dilution with them as a mixture. A standard curve was created using the vitamin D mixture and had an equation of y = 137.08x – 33.06, with a R^2 value of 0.9994, as shown in Graph 7. The standard curve for cholesterol, shown in Graph 8, has an equation of y = 0.7169x + 3.6068, with an R^2 value of 0.9981. The concentrations used in determining the standard curve for all developmental and ranged from 125 µg/mL to 7.81 µg/mL.

The instar samples were analyzed in the HPLC to determine the concentrations of the steroids or vitamins. The chromatogram of the sample and the standard were compared in order to identify the biomolecules in the sample correlating to the standards' retention time. In Figures 8 through 13, the standard of the vitamins/steroids along with the samples show the chromatograms of each. In order to quantify with the use of the standard curves, a standard addition was used. The area determined from the known addition was then subtracted from the area of the peak of the sample containing



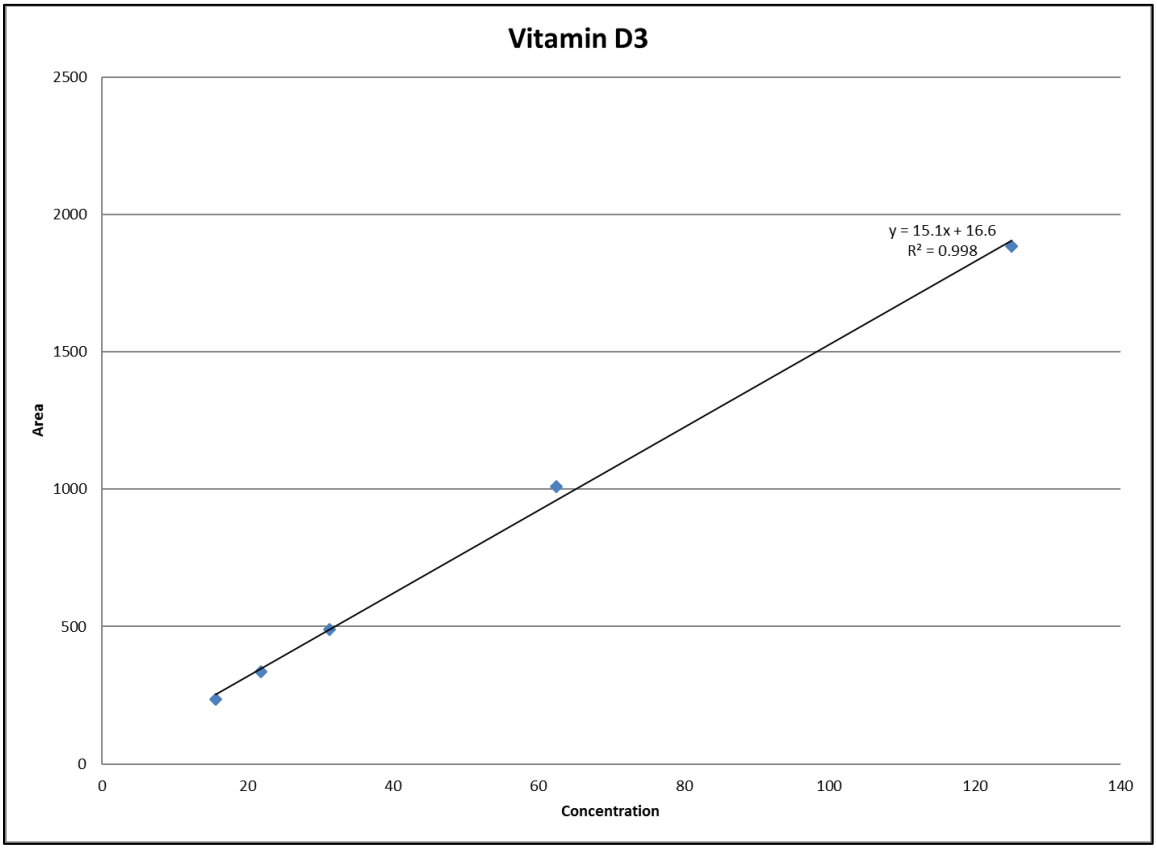
Graph 4: Standard curve of estradiol from HPLC

As concentration of estradiol decreased the area of the peak decreased also. The R^2 value for this line is 0.9998 which shows a good fit for the trendline. The concentrations used for the standard curve ranged from 7.81 µg/mL to 125 µg/mL.



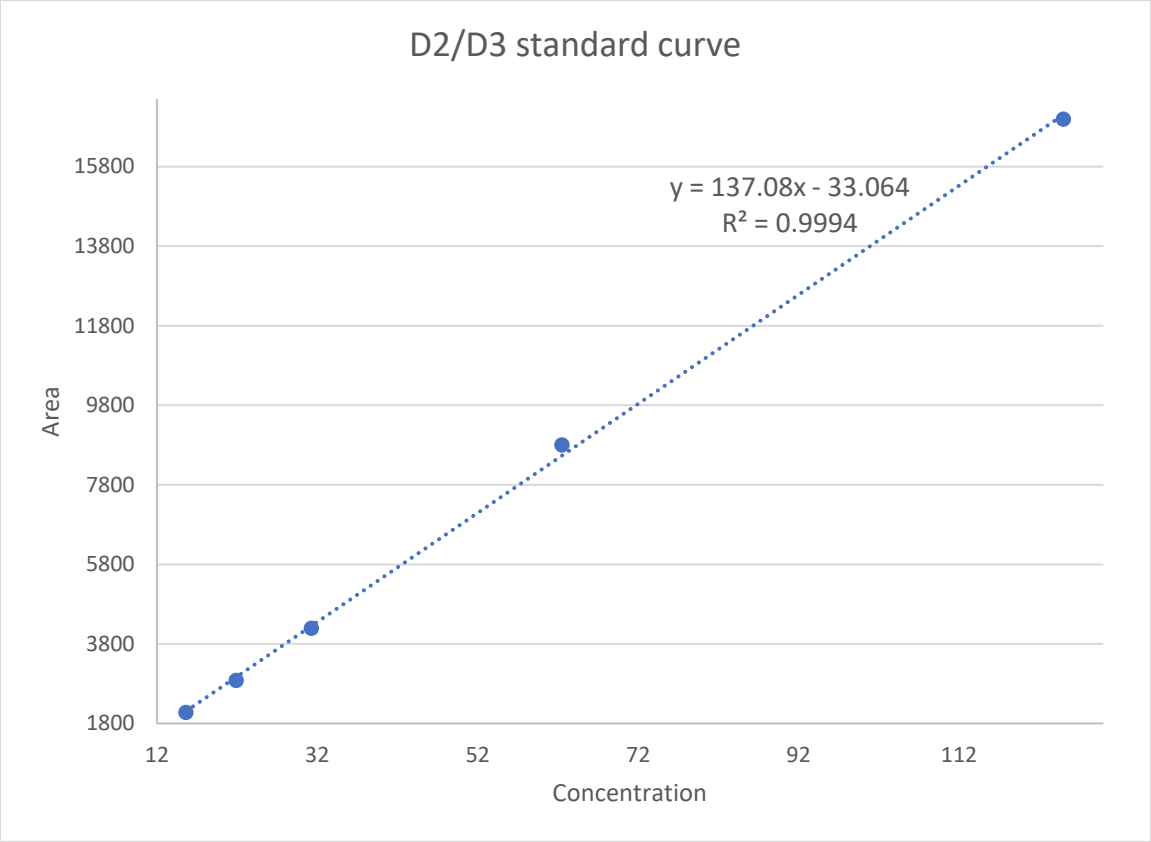
Graph 5: Standard curve of vitamin D_2 from HPLC

As concentration of vitamin D_2 decreased the area of the peak decreased also. The R^2 value for this line is 0.9997 which shows a good fit for the trendline. The concentrations used for the standard curve ranged from 7.81 µg/mL to 125 µg/mL.



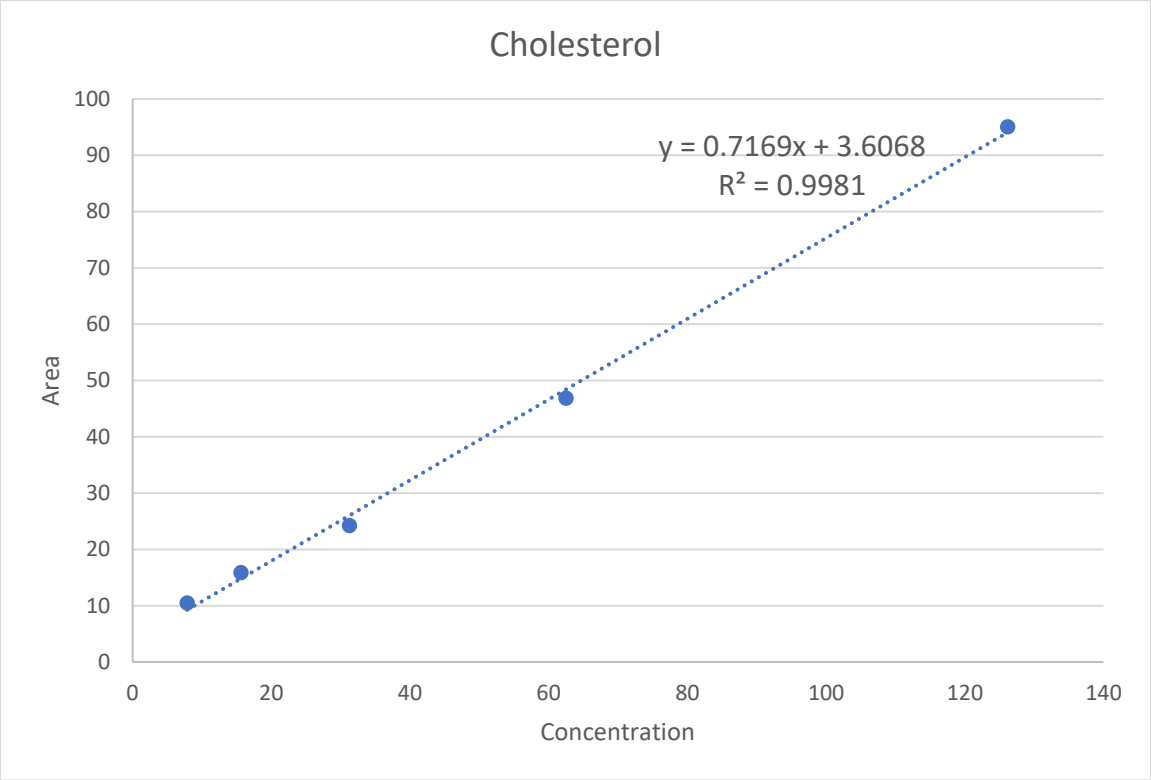
Graph 6: Standard curve of vitamin D_3 from HPLC

As concentration of vitamin D_3 decreased the area of the peak decreased also. The R^2 value for this line is 0.998 which shows a good fit for the trendline. The concentrations used for the standard curve ranged from 7.81 µg/mL to 125 µg/mL.



Graph 7: Standard curve of vitamin D_3 and vitamin D_3 from HPLC

As concentration of vitamin D_3 and vitamin D_3 decreased the area of the peak decreased also. The R^2 value for this line is 0.9994 which shows a good fit for the trendline. The concentrations used for the standard curve ranged from 7.81 µg/mL to 125 µg/mL.



Graph 8: Standard curve of cholesterol from HPLC

As concentration of cholesterol decreased the area of the peak decreased also. The R^2 value for this line is 0.9981 which shows a good fit for the trendline. The concentrations used for the standard curve ranged from 7.81 µg/mL to 125 µg/mL.

the standard addition. The difference in area was used to determine the concentration within the samples. Each sample was analyzed twice in order to produce a standard deviation. Figures 8 and 9 show the HPLC chromatograms of the standard and the sample for estradiol. The standard had a retention time of 3.78 minutes while the sample had a retention time of 3.82 minutes. The change in retention is acceptable since the other molecules within the sample may interact with each other and would account for the 0.04 difference in retention time. The chromatograms in Figures 10 and 11 compare the standard and sample for vitamin D_2 and vitamin D_3. The standard is a mixture of equal parts of vitamin D_2 and D_3 and were compared against the sample since the sample would have a mixture of both. The retention time for the standard of vitamin D_2 and D_3 was determined to be 8.72 minutes and the samples had a retention time of 7.77 minutes. The difference in retention times can be attributed to other molecules within the sample could have interacted with vitamin D allowing it to elude earlier. The retention time for the standard of cholesterol was found to be 8.46 minutes and the sample eluded at 8.16 minutes as shown in Figures 12 and 13.

Estradiol was quantified with three breeding pairs and two sets of siblings for each breeding pair. This allowed for comparison between species and siblings from the same parent. Tables 6 through 10 compares the different concentrations of estradiol found between species at each life stage. Comparing species during their larval

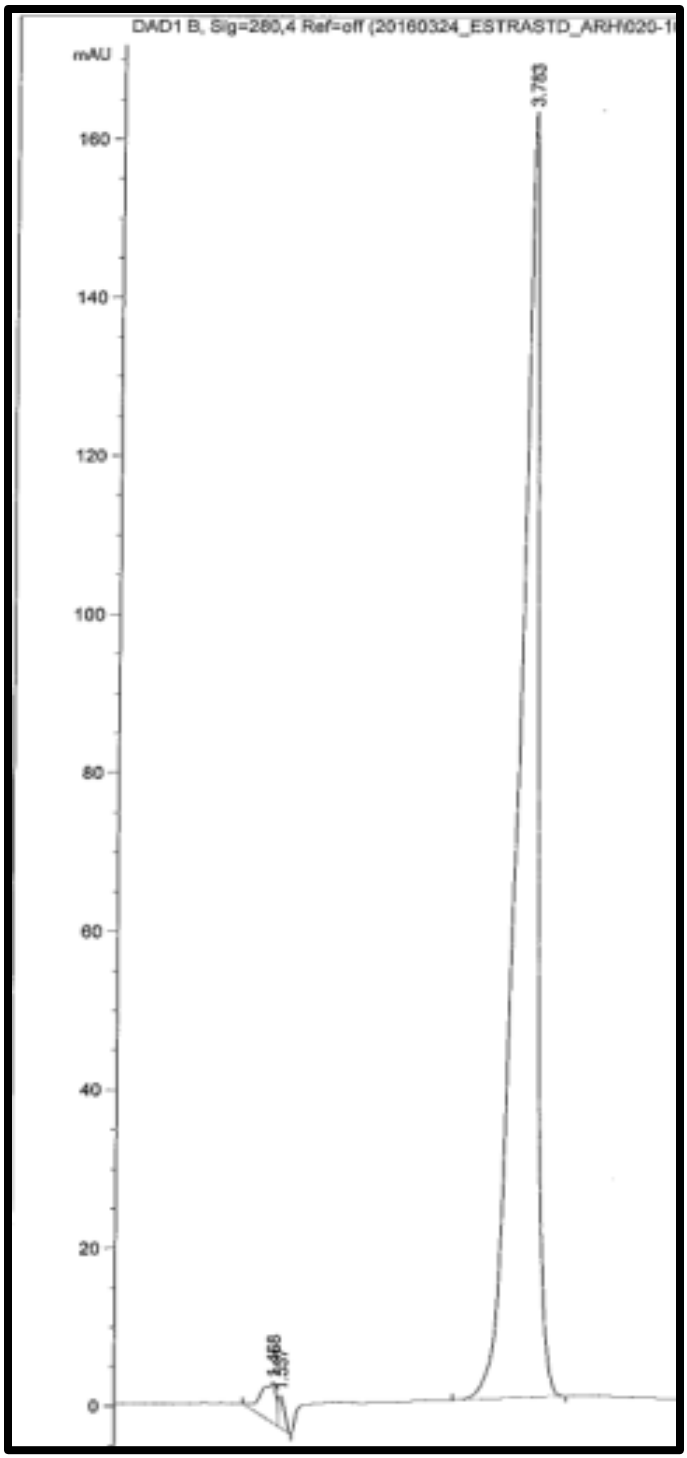


Figure 8: HPLC chromatogram of estradiol standard

Chromatogram from HPLC showing the retention time of 3.78 minutes for a standard of estradiol with a concentration of 125 µg/mL. The column used was a reverse phase with a mobile phase of 0.1% formic acid and MeOH.

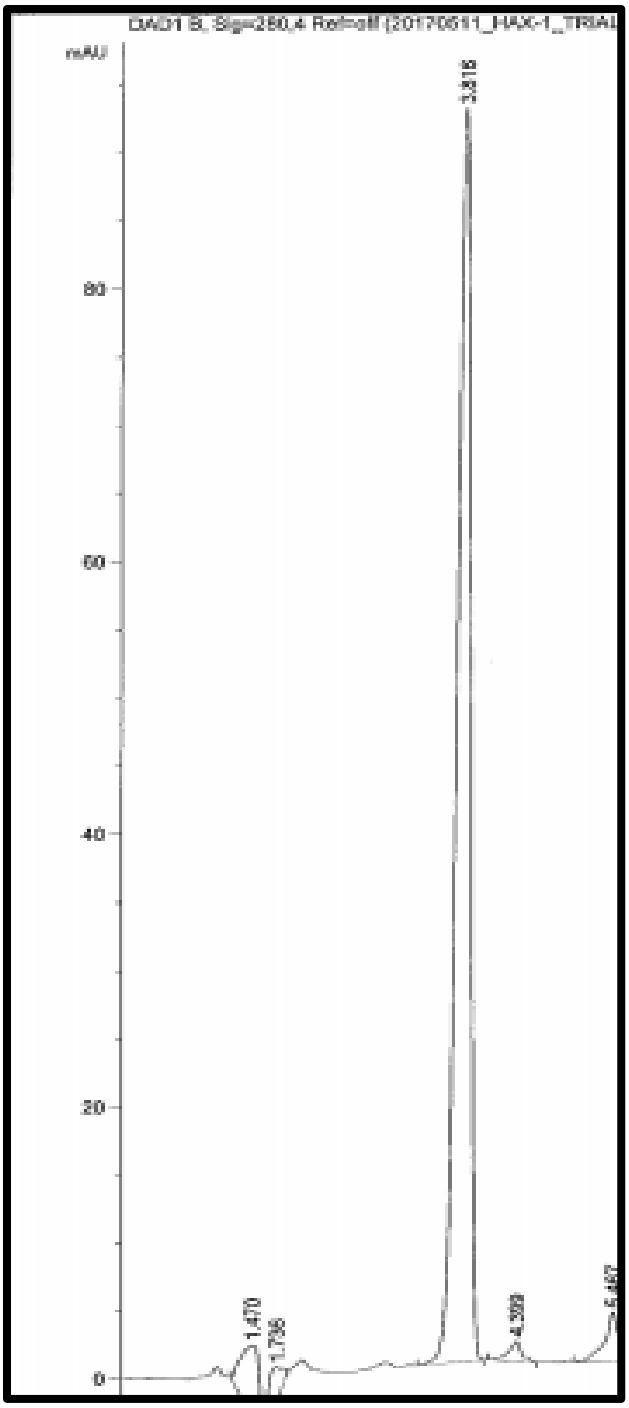


Figure 9: HPLC chromatogram of *H. axyridis* larvae for estradiol

Chromatogram from HPLC showing the retention time of 3.82 minutes which correlates to the standard chromatogram for estradiol. This has a standard addition to allow for quantification.

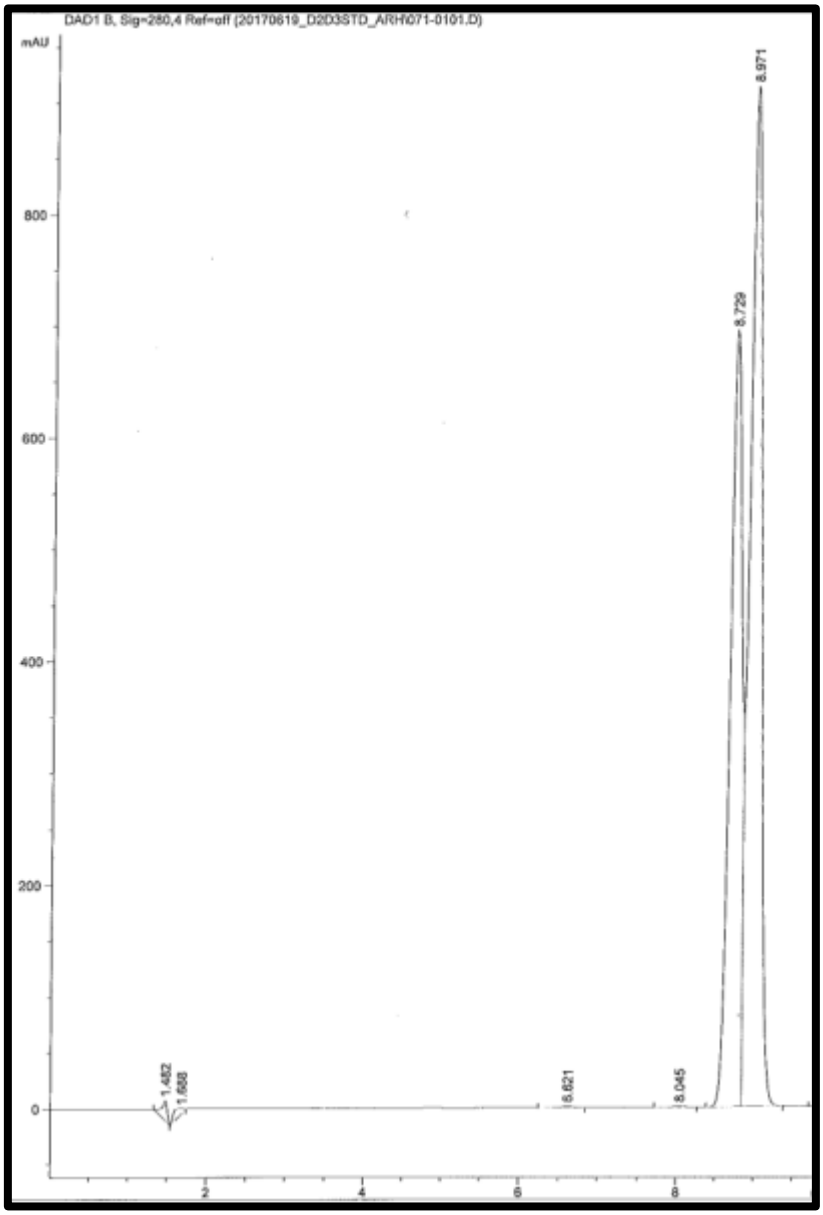


Figure 10: HPLC chromatogram of vitamin D_2 and D_3 standard.

Chromatogram from HPLC showing the retention time of 8.72 minutes for a standard of vitamin D_2 and vitamin D_3 with a concentration of 125 µg/mL. The column used was a C-18 column and had a mobile phase of ACN and MeOH.

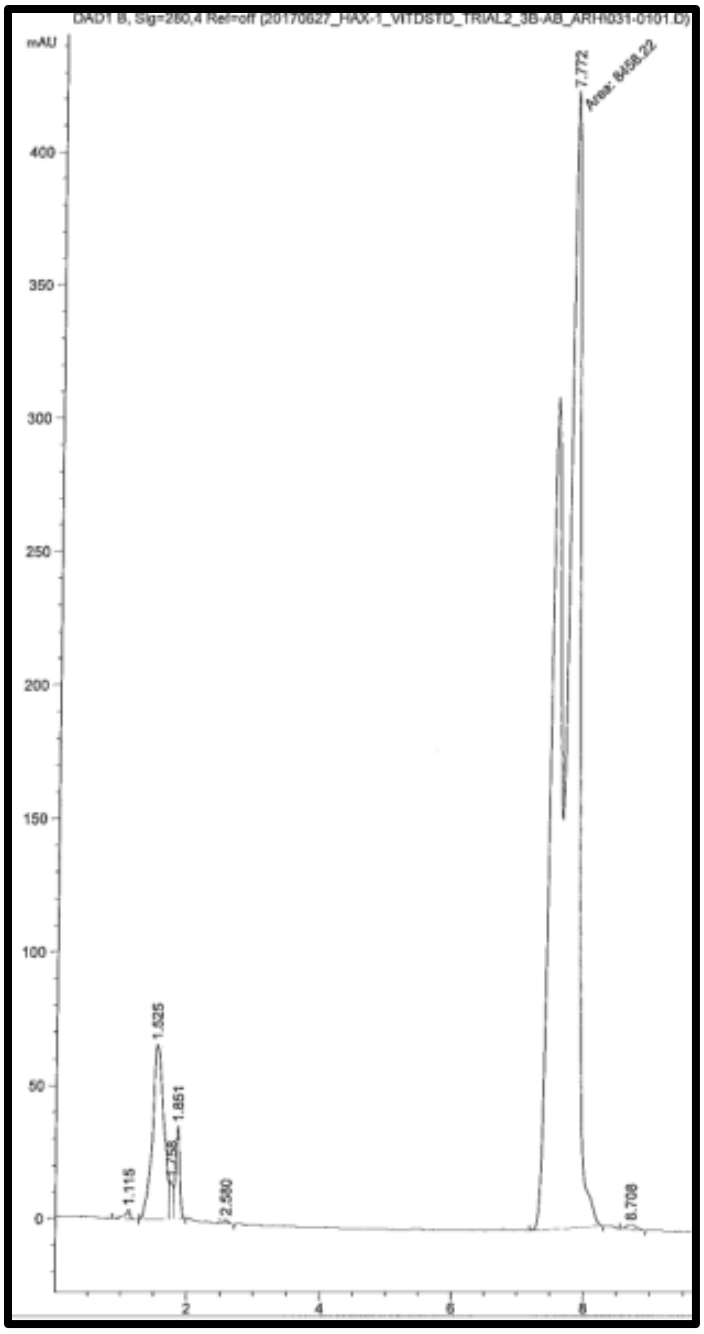


Figure 11: HPLC chromatogram of *H. axyridis* larvae for vitamin D.

It has a retention time of 7.77 minutes which correlates to the standard chromatogram for vitamin D_2 and vitamin D_3. This has a standard addition to allow for quantification.

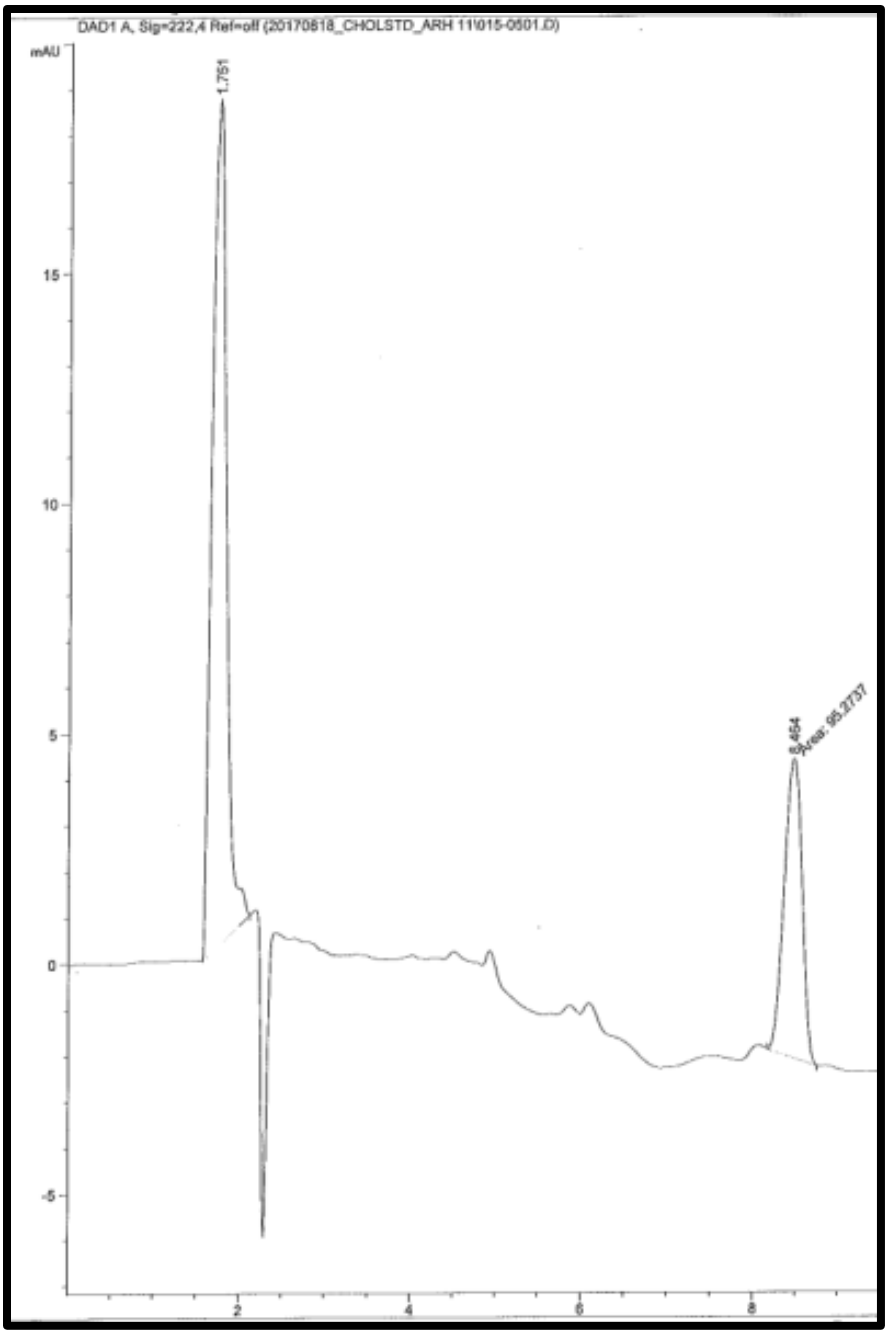


Figure 12: HPLC chromatogram of cholesterol standard.

The chromatogram from HPLC used a reverse phase column and had a mobile phase of ACN and MeOH. The retention time was found to be 8.46 minutes.

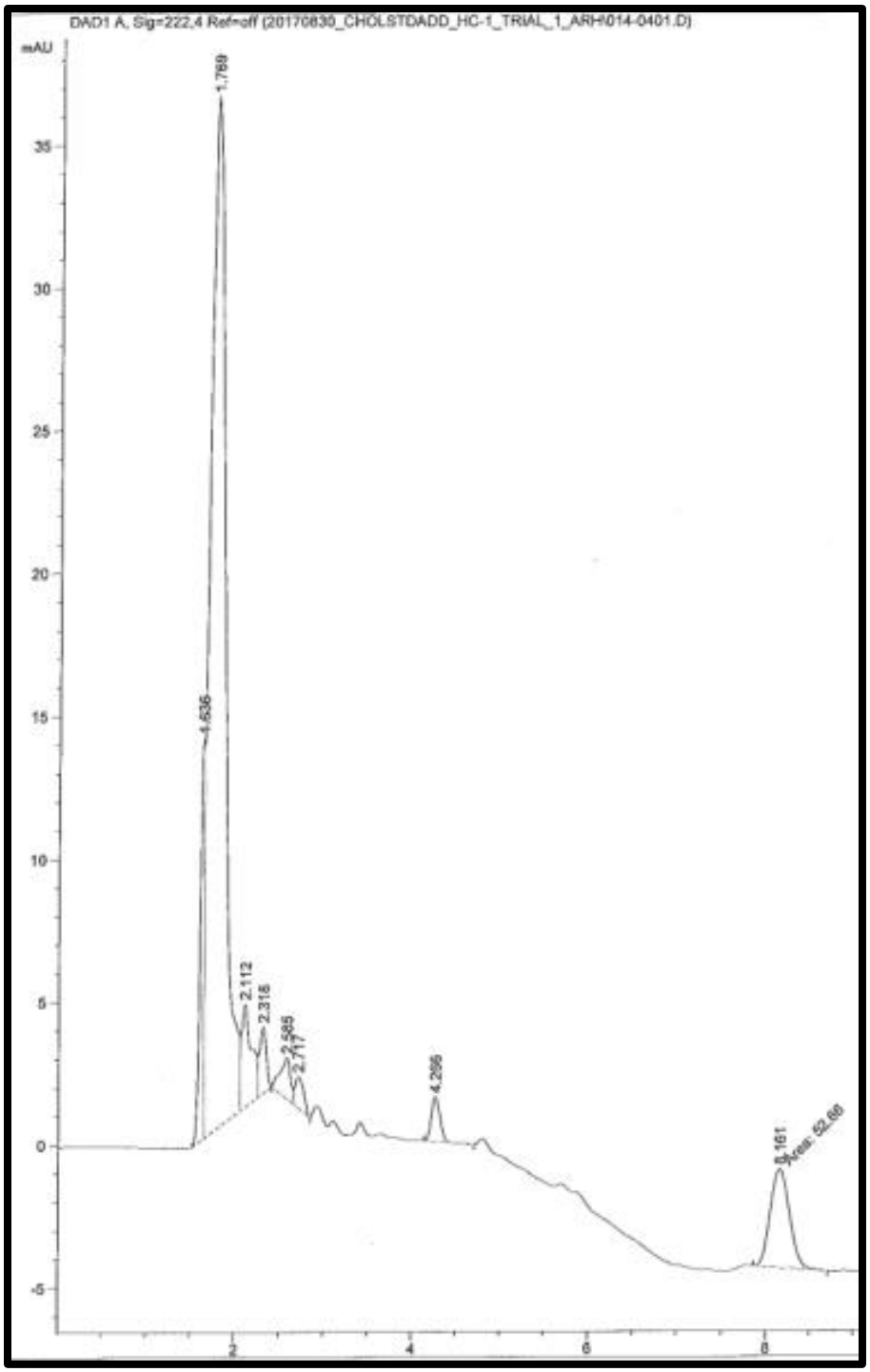


Figure 13: HPLC chromatogram of *H. convergens* larvae for cholesterol

It has a retention time of 8.16 minutes which correlates to the standard chromatogram for cholesterol. This has a standard addition to allow for quantification.

stages there is a 2-fold increase in estradiol in *H. convergens* throughout all the larval stages. The greatest difference was found in the adult stage with a 5-fold increase in *H. convergens* over *H. axyridis* (Table 10). Between the second and third instar stages, it was found to have a 4-fold increase in *H. convergens* and *H. axyridis* was determined to have a 5-fold increase (Table 7 and Table 8). Comparisons between sibling sets of breeding pairs of *H. convergens* and *H. axyridis* are shown in Tables 11 through 16. The concentrations of estradiol between sibling set one is greater than that of sibling set two for the species *H. convergens*, whereas sibling set two is greater than sibling set one in *H. axyridis* (Table 11 through 16). This difference in concentration could be a determination of gender in the different larval stages of the lady beetles.

The concentration of estradiol was used to determine the mass of estradiol in micrograms per milligram of fresh body weight for each sample. The volume of the sample was used to convert the concentration from micrograms per milliliter to micrograms of estradiol and then divided by the weight of the corresponding instar. Comparisons between the sibling sets of each breeding pair are shown in Table 17 through 22. As the larvae develop the mass of estradiol per milligram of body weight decreases for both species. The mass of estradiol was compared for each species and shown in Table 23 through 28 and also show a decrease through their development. The native species *H. convergens* has a concentration of estradiol than the invasive species *H. axyridis* throughout their development with the greatest increase during their adult stage with a 22-fold increase. There is an increase in estradiol in the native species between the second and third instars as well with a 1-fold increase whereas the invasive species has a decrease in estradiol concentration by 1-fold.

Table 6: Comparison of first instars of *H. convergens* and *H. axyridis*

First Instar		
Species	Mean µg/mL	RSD
H. convergens	3.46	0.499
H. axyridis	1.54	1.04

Concentrations of estradiol in two species of predatory lady beetles. There is a 2-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 7: Comparison of second instars of *H. convergens* and *H. axyridis*

Second Instar		
Species	Mean µg/mL	RSD
H. convergens	4.34	0.571
H. axyridis	1.80	0.746

Concentrations of estradiol in two species of predatory lady beetles. There is a 2-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 8: Comparison of third instars of *H. convergens* and *H. axyridis*

Third Instar		
Species	Mean µg/mL	RSD
H. convergens	19.34	0.487
H. axyridis	10.63	0.788

Concentrations of estradiol in two species of predatory lady beetles. There is a 2-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 9: Comparison of fourth instars of *H. convergens* and *H. axyridis*

Fourth Instar		
Species	Mean µg/mL	RSD
H. convergens	23.79	0.512
H. axyridis	12.98	0.720

Concentrations of estradiol in two species of predatory lady beetles. There is a 2-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 10: Comparison of adult *H. convergens* and *H. axyridis*

Adult		
Species	Mean µg/mL	RSD
H. convergens	28.82	0.126
H. axyridis	5.75	1.40

Concentrations of estradiol in two species of predatory lady beetles. There is a 5-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 11: Comparison of siblings through life stages from the first breeding pair of *H. convergens*

H. convergens pair 1				
	Sibling 1		Sibling 2	
Instar	Mean µg/mL	RSD	Mean µg/mL	RSD
1st	6.22	0.176	1.99	0.045
2nd	8.68	0.215	2.02	0.221
3rd	26.48	0.124	10.85	0.199
4th	36.15	0.180	13.26	0.021

Concentrations of estradiol in one breeding pair of *H. convergens*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The larger concentrations of estradiol could be a determinant of the gender of the instars.

Table 12: Comparison of siblings through life stages from the second breeding pair of *H. convergens*

H. convergens pair 2				
	Sibling 1		Sibling 2	
Instar	Mean µg/mL	RSD	Mean µg/mL	RSD
1st	4.31	0.016	1.77	0.197
2nd	4.81	0.074	2.41	0.087
3rd	27.03	0.096	10.85	0.026
4th	35.48	0.141	11.99	0.019

Concentrations of estradiol in one breeding pair of *H. convergens*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The larger concentrations of estradiol could a determinant of the gender of the instars.

Table 13: Comparison of siblings through life stages from the third breeding pair of *H. convergens*

H. convergens pair 3				
	Sibling 1		Sibling 2	
Instar	Mean µg/mL	RSD	Mean µg/mL	RSD
1st	4.33	0.057	2.14	0.255
2nd	5.39	0.158	2.74	0.075
3rd	31.31	0.143	10.17	0.083
4th	34.09	0.118	11.75	0.076

Concentrations of estradiol in one breeding pair of *H. convergens*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The larger concentrations of estradiol could a determinant of the gender of the instars.

Table 14: Comparison of siblings through life stages from the first breeding pair of *H. axyridis*

H. axyridis pair 1				
	Sibling 1		Sibling 2	
Instar	Mean µg/mL	RSD	Mean µg/mL	RSD
1st	ND	ND	2.60	0.203
2nd	0.74	1.17	2.81	0.075
3rd	7.34	1.16	13.60	0.063
4th	11.16	1.02	16.90	0.084

Concentrations of estradiol in one breeding pair of *H. axyridis*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The larger concentrations of estradiol could a determinant of the gender of the instars.

Table 15: Comparison of siblings through life stages from the second breeding pair of *H. axyridis*

H. axyridis pair 2				
	Sibling 1		Sibling 2	
Instar	Mean µg/mL	RSD	Mean µg/mL	RSD
1st	1.17	1.16	3.00	0.301
2nd	0.65	2.00	3.41	0.234
3rd	6.98	2.00	14.31	0.140
4th	8.73	1.17	14.19	0.091

Concentrations of estradiol in one breeding pair of *H. axyridis*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The larger concentrations of estradiol could a determinant of the gender of the instars.

Table 16: Comparison of siblings through life stages from the second breeding pair of *H. axyridis*

H. axyridis pair 3				
	Sibling 1		Sibling 2	
Instar	Mean µg/mL	RSD	Mean µg/mL	RSD
1st	ND	ND	2.45	0.204
2nd	0.30	1.31	2.88	0.011
3rd	7.88	1.21	13.68	0.029
4th	10.59	1.02	16.32	0.558

Concentrations of estradiol in one breeding pair of *H. axyridis*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The larger concentrations of estradiol could a determinant of the gender of the instars.

Table 17: Mass of estradiol per milligram of body weight of siblings through life stages from the first breeding pair of *H. convergens*

H. convergens pair 1				
	Sibling 1		Sibling 2	
Instar	Mean µg/mg	RSD	Mean µg/mg	RSD
1st	25.90	0.176	8.27	0.045
2nd	8.58	0.215	1.99	0.221
3rd	7.70	0.124	3.15	0.199
4th	3.35	0.180	1.23	0.021

Comparing mass of estradiol per body weight of each life stage of *H. convergens*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The mass of estradiol per body weight decreases through each life stage except for sibling set 2 which has an increase in estradiol from second to third instar.

Table 18: Mass of estradiol per milligram of body weight of siblings through life stages from the second breeding pair of *H. convergens*

H. convergens pair 2				
	Sibling 1		Sibling 2	
Instar	Mean µg/mg	RSD	Mean µg/mg	RSD
1st	12.96	0.016	5.32	0.197
2nd	6.31	0.074	3.16	0.087
3rd	8.53	0.096	3.21	0.026
4th	2.46	0.141	0.83	0.019

Comparing mass of estradiol per body weight of each life stage of *H. convergens*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The mass of estradiol per body weight decreases through each life stage except for sibling set 2 which has an increase in estradiol from second to third instar.

Table 19: Mass of estradiol per milligram of body weight of siblings through life stages from the third breeding pair of *H. convergens*

H. convergens pair 3				
	Sibling 1		Sibling 2	
Instar	Mean µg/mg	RSD	Mean µg/mg	RSD
1st	15.67	0.057	7.76	0.255
2nd	5.04	0.158	2.57	0.075
3rd	10.71	0.143	3.48	0.083
4th	2.49	0.118	0.86	0.076

Comparing mass of estradiol per body weight of each life stage of *H. convergens*. Sibling set 1 has a greater concentration of estradiol than sibling set 2. The mass of estradiol per body weight decreases through each life stage except for an increase in estradiol from second to third instar.

Table 20: Mass of estradiol per milligram of body weight of siblings through life stages from the first breeding pair of *H. axyridis*

H. axyridis pair 1				
	Sibling 1		Sibling 2	
Instar	Mean µg/mg	RSD	Mean µg/mg	RSD
1st	ND	ND	9.91	0.203
2nd	0.18	1.17	3.16	0.075
3rd	0.55	1.16	3.18	0.063
4th	0.39	1.02	0.80	0.084

Comparing mass of estradiol per body weight of each life stage of *H. axyridis*. Sibling set 2 has a greater concentration of estradiol than sibling set 1. The mass of estradiol per body weight decreases through each life stage except for an increase in estradiol from second to third instar.

Table 21: Mass of estradiol per milligram of body weight of siblings through life stages from the second breeding pair of *H. axyridis*

H. axyridis pair 2				
	Sibling 1		Sibling 2	
Instar	Mean µg/mg	RSD	Mean µg/mg	RSD
1st	1.19	1.16	9.70	0.301
2nd	0.13	2.00	5.69	0.234
3rd	0.24	2.00	4.02	0.140
4th	0.15	1.17	0.83	0.091

Comparing mass of estradiol per body weight of each life stage of *H. axyridis*. Sibling set 2 has a greater concentration of estradiol than sibling set 1. The mass of estradiol per body weight decreases through each life stage except for an increase in estradiol from second to third instar in sibling set 1.

Table 22: Mass of estradiol per milligram of body weight of siblings through life stages from the third breeding pair of *H. axyridis*

H. axyridis pair 3				
	Sibling 1		Sibling 2	
Instar	Mean µg/mg	RSD	Mean µg/mg	RSD
1st	ND	ND	9.74	0.204
2nd	0.06	1.31	3.20	0.011
3rd	0.69	1.21	2.69	0.029
4th	0.33	1.02	1.06	0.558

Comparing mass of estradiol per body weight of each life stage of *H. axyridis*. Sibling set 2 has a greater concentration of estradiol than sibling set 1. The mass of estradiol per body weight decreases through each life stage except for an increase in estradiol from second to third instar in sibling set 1.

Table 23: Mass of estradiol per milligram of body weight of first instars *H. convergens* and *H. axyridis*

First Instar		
Species	Mean µg/mg	RSD
H. convergens	12.65	0.582
H. axyridis	7.64	0.993

Concentrations of estradiol in two species of predatory lady beetles. There is a 2-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 24: Mass of estradiol per milligram of body weight of second instars *H. convergens* and *H. axyridis*

Second Instar		
Species	Mean µg/mg	RSD
H. convergens	4.61	0.538
H. axyridis	2.07	1.07

Concentrations of estradiol in two species of predatory lady beetles. There is a 2-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 25: Mass of estradiol per milligram of body weight of third instars *H. convergens* and *H. axyridis*

Third Instar		
Species	Mean µg/mg	RSD
H. convergens	6.13	0.954
H. axyridis	1.89	1.56

Concentrations of estradiol in two species of predatory lady beetles. There is a 3-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 27: Mass of estradiol per milligram of body weight of fourth instars *H. convergens* and *H. axyridis*

Fourth Instar		
Species	Mean µg/mg	RSD
H. convergens	1.87	0.540
H. axyridis	0.59	0.746

Concentrations of estradiol in two species of predatory lady beetles. There is a 3-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Table 27: Mass of estradiol per milligram of body weight of adult *H. convergens* and *H. axyridis*

Adult		
Species	Mean µg/mg	RSD
H. convergens	1.19	0.25
H. axyridis	0.055	1.44

Concentrations of estradiol in two species of predatory lady beetles. There is a 22-fold increase in estradiol production from *H. convergens* over *H. axyridis*. Both species were developed under the same conditions and fed the same diet of fava bean aphids.

Identification of vitamin D_2, vitamin D_3, or cholesterol with the use of HPLC was accomplished using a modified protocol (Turpeinen, 2003). The concentrations of vitamins D_2 and D_3 were found to be in the tenths of micrograms or below the limit of detection of the instrument. This was also seen with cholesterol quantification in the samples. In third and fourth instars, tenths of micrograms per milliliter of cholesterol were isolated, except in first through third instars where the concentrations of cholesterol were below the limit of detection of the instrument.

Gas chromatography-mass spectroscopy was used to verify the identification of the steroids and vitamins. Samples from each of the species though all of their life stages were analyzed with GC-MS. Estradiol was found to have a retention time of 21.85 minutes for the standard and the samples. Vitamin D_2 and vitamin D_3 standards were analyzed in a mixture and had retention times of 23.04 and 22.78 minutes respectively. The chromatogram for cholesterol was analyzed and yielded a retention time of 23.07 minutes. The corresponding spectrums from the mass spectroscopy were used to determine the fragments of each of the vitamins and steroids. The spectrums for the vitamins and steroids are shown in Figures 14 through 17.

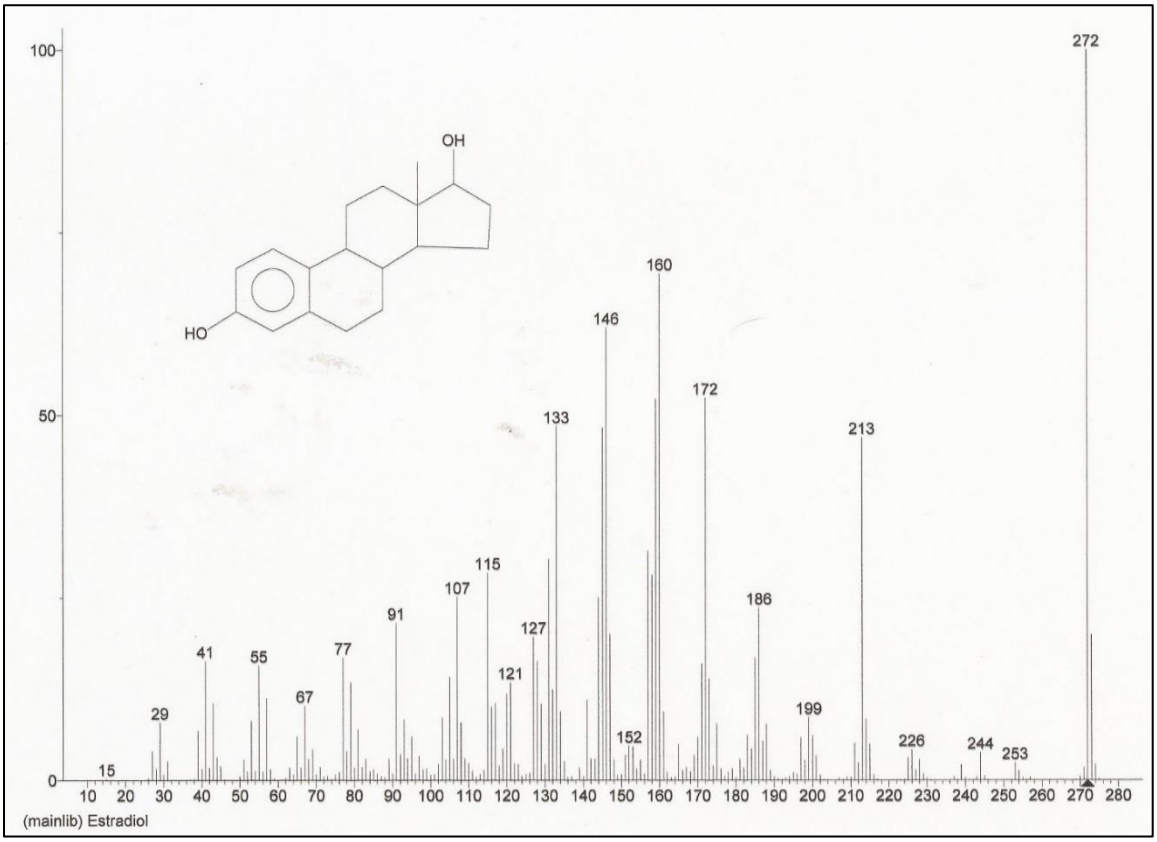


Figure 14: Spectrum of estradiol from GC-MS

The spectrum was found using a temperature ramp from 60.0°C to 325°C in 26 minutes, with an injection volume of 4 µL. Both the standard and the samples used this protocol.

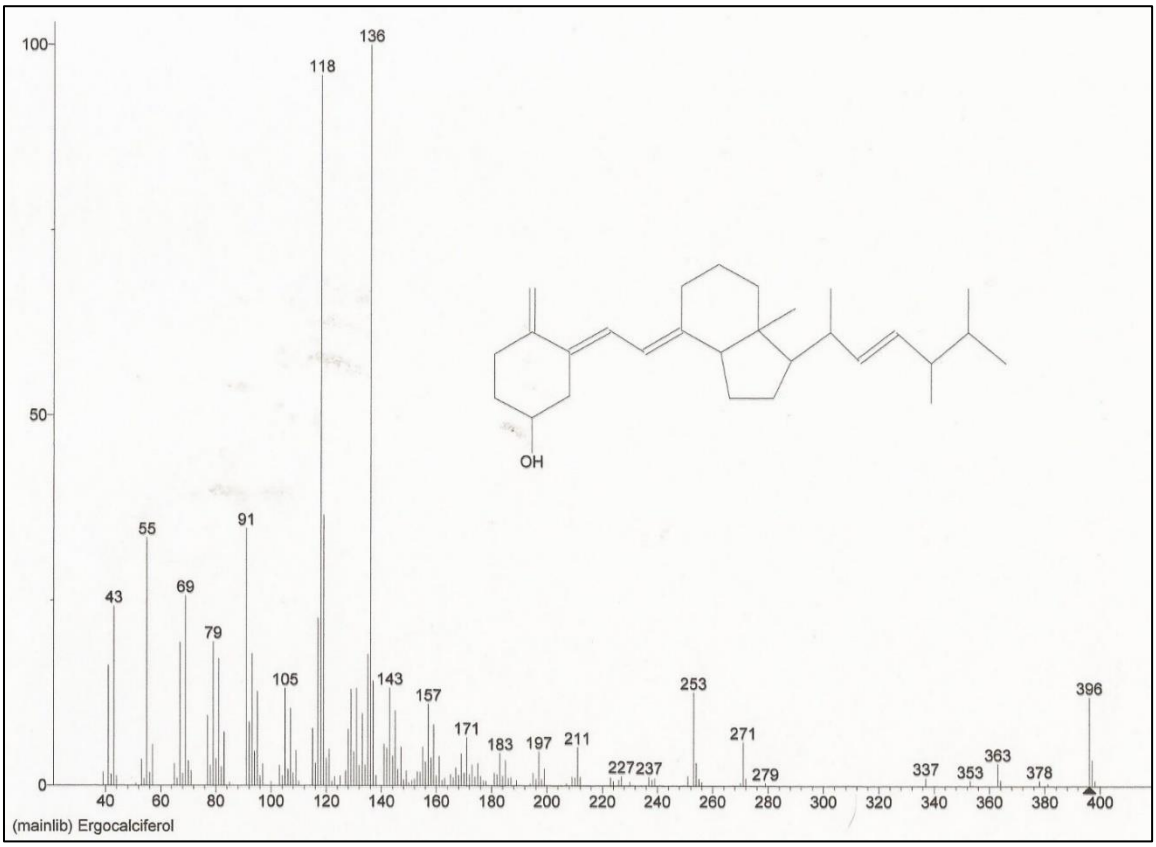


Figure 15: Spectrum of vitamin D_2 from GC-MS

The spectrum was found using a temperature ramp from 60.0°C to 325°C in 24 minutes, with an injection volume of 4 µL. Both the standard and the samples used this protocol.

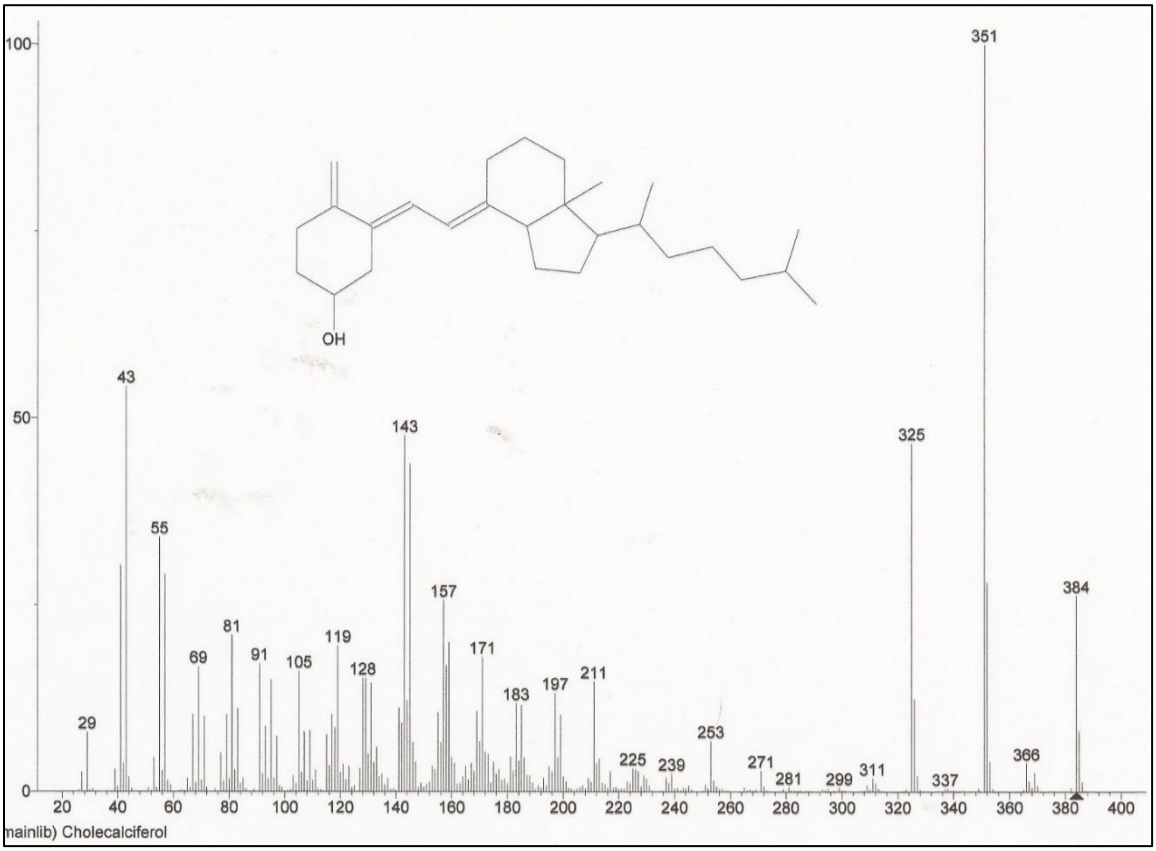


Figure 16: Spectrum of vitamin D_3 from GC-MS

The spectrum was found using a temperature ramp from 60.0°C to 325°C in 24 minutes, with an injection volume of 4 µL. Both the standard and the samples used this protocol.

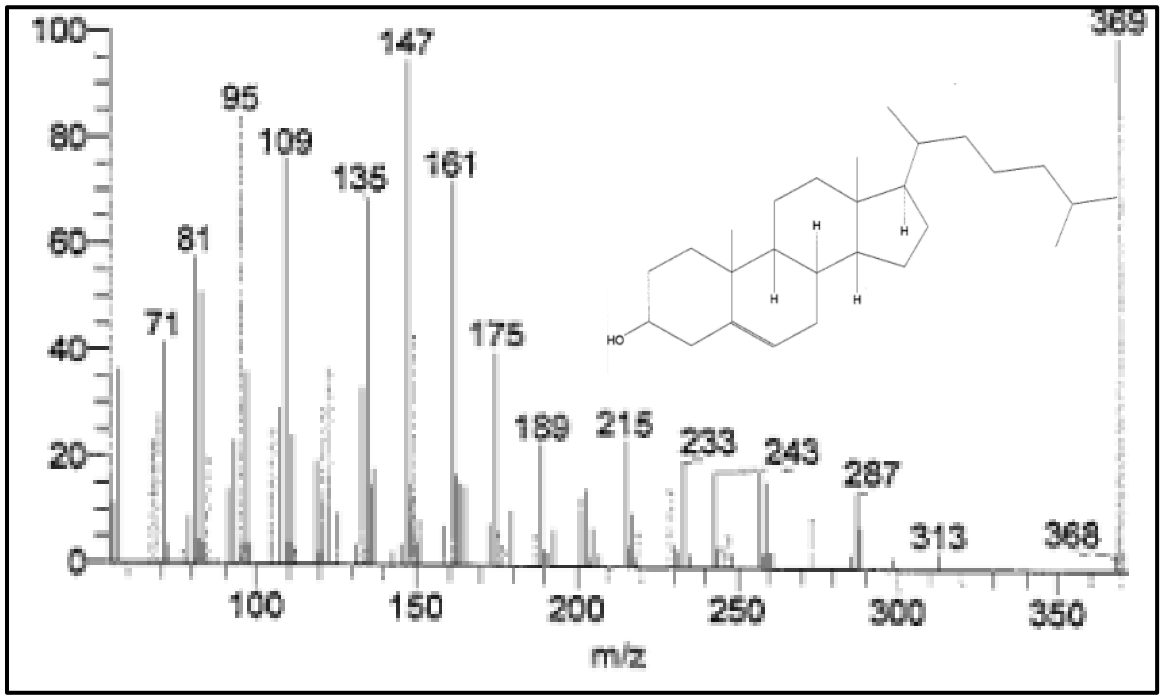


Figure 17: Spectrum of cholesterol from GC-MS

The spectrum was found using a temperature ramp from 60.0°C to 325°C in 24 minutes, with an injection volume of 4 µL. Both the standard and the samples used this protocol.

Vitamin D and cholesterol were identified on GC-MS and HPLC; however, concentrations of these molecules were determined to be in the tenths of micrograms per milliliter to not detectable. By using multiple instruments, the identification of the developmental vitamins and steroids can be confirmed within the samples. It is of interest that these developmental biomolecules are present and can be detected from samples with small physical sizes.

DISCUSSION

Since few studies have been conducted on the identification of vitamins and steroids, the purpose for this research is to determine the quantities of these vitamins and steroids to find a way to effectively manage the populations without the use of harmful pesticides. The results of this research indicate that a disruption in the production of estradiol between the second and third instars could delay the development of the larvae to the adult stage. The identification of vitamin D and cholesterol could provide a second pathway to disrupt their development as well.

The production of estradiol was at its highest concentration for the invasive species *H. axyridis*, between the second and third instars showing a 6-fold increase. The native species *H. convergens* demonstrated a 4-fold increase between the second and third instars. It is also interesting to note the difference in concentration of estradiol between the species in their adult stage. The species C*onvergens* had a 5-fold increase over *axyridis*. This is interesting because *H. axyridis* has a greater population size than *H. convergens* (Evans et al, 2011; Majerus et al, 2006). Sets of siblings were compared as well, and found that those from the same parents showed significantly different concentrations of estradiol which has not been seen before. This could determine the gender of the instars, since there were no other methods to distinguish between genders. The lady beetles were fed a diet of fava bean aphids exclusively. It could be possible that their diet had an impact on their rate of reproduction and production of estradiol. Kajita's (2009) study on the effect of reproduction of lady beetles and their diet. When their diet was changed, the results found that their size and quantity of the eggs production was affected in the native species. It was shown that the invasive species experienced less

stress when their feeding substance was changed. If the food source is limited than it might allow the invasive to still thrive but the native species would not be able to reproduce as effectively (Kajita et al, 2009). If the food source was changed, this could lead to a lower concentration of estradiol and other developmental steroids as well as vitamins necessary for reproduction and growth.

The comparison of estradiol per fresh body weight yielded a decreasing concentration of estradiol. As the lady beetle increases in mass the mass of estradiol decreases. When the first instars hatch from their eggs they may have a higher concentration of estradiol from their food source within the yolk of the egg. After hatching their food source changed to fava bean aphids which could alter the amount of estradiol that could be synthesized. They could also need less estradiol as the mature and need more of another developmental molecule which would lead to lower concentrations per body mass of estradiol.

The steroid induced carrier protein that is involved in yolk production known as vitellogenin (Vg) plays a key role in reproduction (Chapin and Brou, 1991; Gordon et al, 1985; Meinwald et al, 1985; Schildknecht, 1970; Tufail et al, 2014). The juvenile hormone along with estrogens are responsible for the transcription of the precursor gene of vitellogenin and are not sex specific (Tufail et al, 2014; Wiley et al, 1981). The protein allows nutrients, carbohydrates and lipids, to be carried to the ovary for yolk production. It would be interesting to see if there is a relationship in the increase of the protein to the concentration of cholesterol and estradiol since cholesterol is a precursor to estradiol. If the synthesis of Vg is disrupted by the lack of developmental steroids either through their diet or by introducing a synthetic form of the steroid, yolk production could be delayed.

This could then lower the population if unviable eggs are produced as offspring could not survive.

Due to the origin of the invasive species, *H. axyridis*, which spans from east Asia to Japan, this species of lady beetle could survive a wide range of habitats (Majerus et al, 2006). This has allowed them to colonize most of Canada, United States, South America, and Europe (Evans et al, 2011; Majerus et al, 2006). Greenhouse pesticides have been used to limit population sizes of lady beetles but have some environmental and human health effects (Damalas et al, 2011; Youn et al, 2003). By looking at developmental steroids and vitamins of lady beetles, it could limit the amount of pesticides used by controlling their maturation by control their ability to synthesize the hormones and vitamins.

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