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Daniel L. Allen Jr.  
*Georgia Southern University*

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**Carbon dioxide sensitivity in two disjunct populations of the pitcher-plant mosquito,  
*Wyeomyia smithii* (Diptera: Culicidae)**

An Honors Thesis submitted in partial fulfillment of the requirements for Honors in the  
Department of Biology.

By:

Daniel Allen

Under the mentorship of Dr. William S. Irby

**Abstract:**

The pitcher-plant mosquito, *Wyeomyia smithii*, utilizes carbon dioxide receptors primarily on their maxillary palps to seek potential hosts for blood meals. Two disjunct populations of *W. smithii* were analyzed to test for differences in carbon dioxide sensitivity that would correlate to varying levels of autogeny, ranging from the autogenous Northern populations (from North Carolina through Canada) to the anautogenous Southern populations (Florida – Louisiana), with the Georgia population exhibiting a shift from autogeny to anautogeny over the past two decades. I compared Georgia (Tattnall Co.) and Florida populations using blood feeding assays and olfactometry assays. Willingness to blood feed was assessed using hand-in-cage assays, and olfactometry assays were conducted using a box dual-choice olfactometer to determine decision-making when exposed to a carbon dioxide source. Results demonstrated that the Southern population was more likely to take a blood meal than the Tattnall County population and that the Southern population has a higher sensitivity to carbon dioxide than the Tattnall County population. This may be explained by differences in environmental conditions between the two habitats.

Thesis Mentor \_\_\_\_\_

Dr. William S. Irby

Honors Director \_\_\_\_\_

Dr. Steven Engel

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Biting insects are a topic that most people have had experience with. Particularly of interest are Diptera species that seek blood meals. One thing that always comes to mind when discussing blood-feeding insects is vector-borne disease, and many Diptera offer examples of these diseases: bluetongue virus transmitted by *Culicoides* midges (Burgin, et al., 2013), malaria transmitted by *Anopheles* mosquitoes, and leishmaniasis transmitted by *Phlebotomus* (phlebotomine) sand flies. These, and many other flies, are spread throughout the world and transmit many other viruses and parasites.

A key group of the biting insects are the Culicidae, more commonly known as mosquitoes. There are “about 34 genera with 3500 species” in this group. The mosquitoes are found throughout the world, often carrying disease and death wherever they are found (Venkateswarlu, Kiran, & Eswari, 2012). To the mosquito, this is a side-effect of its drive to reproduce. Female mosquitoes that take blood meals do so in order to acquire proteins from serum for egg production (Emami, Ranford-Cartwright, & Ferguson, 2013). Male mosquitoes do not exhibit the blood-feeding behavior. Mosquitoes have to find an adequate host in order to blood feed. This requires a variety of attractants, ranging from chemicals to visual and physical cues. Carbon dioxide, water vapor, temperature, and many other stimulants can induce host-seeking and blood-feeding in many mosquitoes (Klun, Kramer, & Debboun, 2013). For this study, the effects of carbon dioxide were studied.

There are many species of mosquitoes in our world, but a novel species that exhibits significant variation in blood-feeding behavior found in North America is *Wyeomyia smithii*, the pitcher-plant mosquito, which lives in association with the purple pitcher-plant, *Sarracenia purpurea*. The larvae and pupae of *W. smithii* live as

commensal organisms in the pitchers of *S. purpurea* (Kneitel & Miller, 2002). *Wyeomyia smithii* occurs exclusively within the range of *S. purpurea*, extending from the Gulf Coast along the eastern seaboard and throughout the Canadian Shield (Figure 1). These populations are nearly always isolated from one another, as *W. smithii* demonstrates poor flight abilities (Merz, et al., 2013). This has led to the development of unique characteristics between populations.

Blood-feeding behavior in *W. smithii* exists in two forms: obligate autogeny and facultative autogeny. Obligate autogeny refers to the northern populations of *W. smithii* where the females will not take a blood meal after the initial egg batch (Clements, 1992). Facultative autogeny means that the female mosquito may take a blood meal after the initial egg batch; the southern populations fall into this category. From previous work in our laboratory, a third population, located in Tattnall County, Georgia, has exhibited a shift in blood-feeding behavior over the past two decades of study. The Tattnall County population was historically obligatorily autogenous but has been observed to blood-feed at increasingly higher rates during blood-feeding trials conducted periodically since 2004. This study compares the Tattnall County population and the Southern populations, represented by a population in the Apalachicola National Forest, Florida. If the Southern populations are more likely to blood feed, then they will be more responsive to CO<sub>2</sub> stimulation, and if the Tattnall County population has continued shifting towards the Southern populations, then they will exhibit similar blood feeding and CO<sub>2</sub> response behaviors. Because of the differences in observed blood-feeding behavior, it was hypothesized that there would be a difference in carbon dioxide sensitivity between the Tattnall County population and the Apalachicola National Forest population.

## **MATERIALS AND METHODS**

### **Establishment of colonies**

Colonies of each population of *W. smithii* were established so supplies of mosquitoes were readily available. Two sites were selected based on use in previous studies: Apalachicola National Forest ((30° 16' 57.15", -84° 50' 29.10") and a site in Tattnall County, Georgia (32° 10' 4.81", -82° 0' 36.29"). These sites represent the Southern populations of *W. smithii* and the intermediate population in Georgia.

At each site, larvae were obtained from the pitchers of *S. purpurea* using flexible plastic pipettes and transported in 50 mL centrifuge tubes in a cooler containing freezer packs. Multiple pitchers from multiple plants were sampled at both locations so that heterogeneous samples were obtained at each site. Once at the Georgia Southern University Insectary, the larvae were kept in water in glass bowls of 13 centimeter diameter; crushed Tetramin fish flakes were fed to the larvae as needed. Larvae were allowed to develop into pupae, which were then removed using plastic pipettes and placed in small beakers. These beakers were then placed in Bioquip cubic foot mosquito cages, where they developed into adults. The adults were given access to fresh water bowls to lay eggs in and 10% sugar water in plastic containers with a cotton wick extending from the container was provided. When larvae were detected in the water bowls, the bowl was removed and labeled, and the larvae were maintained as previously described. All life stages of the mosquitoes were kept at 26°C and 80% relative humidity; with a 24 L: 0D light regimen.

**Blood-feeding Assays**

To determine the proportion of females that would feed on blood, hand-in-cage assays were performed. These assays were conducted by having a volunteer place their exposed hand into the cage, with the hand in a relaxed, palm-down position. Two different volunteers were used to collect blood-feeding data. The assays lasted for ten minutes, with the time being marked when a mosquito landed, began feeding, and flew away. An estimate of the total mosquitoes in the cage was determined and divided by two to give an approximate number of female mosquitoes. Assays were done periodically throughout the year. These results were then compared with blood-feeding assays that had been conducted periodically since 1998.

**Olfactometry Assays**

Olfactometry assays were conducted using a 2 ft. x1 ft. x1 ft. dual-choice olfactometer that was constructed using ¼” plexiglass and silicone. A 1 ft. square divider was placed in the center of the enclosure against the back all, creating two equal sides. Dry yeast was placed into 50 mL of a 20% sugar water solution to supply a source of carbon dioxide. A control of 50 mL of 20% sugar water was used as the alternative choice. The yeast solution was placed randomly on one side of the other; a coin was flipped to determine in which side the yeast was put. Three female mosquitoes were removed from a population, one at a time, checked under a microscope to ensure sex, and placed into a large pill container until all three were collected. The pill container was placed into the olfactometer and opened as a clock was started. The times were marked, along with choice of side, when a mosquito flew past the barrier. Once all three mosquitoes had made a choice, or ten minutes had elapsed, the three mosquitoes were

recovered using an aspirator, replaced into the pill container, and a second trial was conducted. Both populations went through this process four times, giving eight total trials per population. Trials from both populations were conducted back-to-back.

## **RESULTS**

### **Blood-Feeding Results**

Blood-feeding of mosquitoes from the Tattnall Co., Georgia and Apalachicola populations has been measured for the past 18 years (Table 1). Large standard deviations indicate a large degree of variability in the blood-feeding behavior of the mosquitoes. A trend emerged over the next four years. There was an increase in both populations' blood-feeding behavior by at least 16%. The assays conducted this year were not an extension of the previous trend, as both populations saw a reduction in the average percent of blood-feeding, with the Tattnall population reverting to levels around the 2007 samples (3.3% in 2015 versus 3.8% in 2007) and the Apalachicola population returning to the 2008 levels (14.8% in 2015 versus 13.8% in 2008).

### **Olfactometry Results**

Once olfactometry results were compiled, percentages of each choice from each population were determined (Fig. 2). The data shows that the Southern populations are more likely to respond to carbon dioxide stimuli than the Tattnall County population. Pair-wise comparisons were made between the choices for carbon dioxide and the control between and within the populations (Table 2). These comparisons were analyzed using Chi-square tests. Two of the four comparisons yielded significant results. The comparison between the carbon dioxide choices between the two populations gave a significant p-value of 0.0166 showing that reactions to carbon dioxide were unequal. The



comparison between the Apalachicola carbon dioxide choices and the Apalachicola control choices generated a significant p-value of 0.006, indicating that the Apalachicola population is more responsive to a stimulus than to no stimulus.

## **DISCUSSION**

Blood-feeding behavior varies immensely in mosquitoes. Most mosquitoes are anautogenous, requiring blood meals for every egg batch produced. Some species, such as *W. smithii*, exhibit various levels of autogeny, as previously discussed. In these autogenous mosquitoes, the first egg batch is always laid without requiring a blood meal. After that, there are two options. The mosquito can be obligatorily autogenous, having the ability to lay additional eggs without blood-feeding, or the mosquito can be facultatively autogenous, taking a blood meal to produce more than one egg batch (Mori, Romero-Severson, Black, & Severson, 2008). Throughout the larval life cycle, the mosquito larvae accumulate nutrients, such as lipids, carbohydrates, and amino acids, which are carried over into the adult stage. In particular, degree of autogeny in pitcher plant mosquitoes is correlated with the quantity of large storage proteins, hexamerins, that accumulate during larval development (Reddy, 2008). These nutrients allow for oogenesis in the autogenous mosquito (Clements, 1992). Autogeny (and fecundity) also depend on the density of larvae during development. As the density increases, there would be fewer nutrients for each individual. This causes smaller egg batches when oogenesis occurs. Because of the lack of nutrients, autogeny is reduced because oogenesis is less likely to occur without a blood meal (Lounibos, Dover, & O'Meara, 1982). As such, it can be seen that the level of autogeny is dependent upon many different factors.

With the pitcher-plant mosquito, *Wyeomyia smithii*, autogeny varies geographically. Northern populations are obligatorily autogenous, never taking a blood meal, while southern populations will seek a blood meal, indicating facultative autogeny (Reddy, 2008). Through this study, we were able to look at both the blood-feeding behavior and the sensitivity to carbon dioxide, a common attractant for blood-feeding arthropods. In past studies, the southern populations would blood-feed after an initial egg batch had been laid. This indicates that the southern species demonstrated and continue to demonstrate, facultative autogeny. The northern populations that had been tested, represented by the populations at the Highlands Biological Research Station in North Carolina, consistently demonstrated obligatory autogeny, never showing any interest in blood meals. Interestingly, the third population found in Tattnall County, Georgia, seemed to shift from obligatory autogeny to facultative autogeny (Table 1).

Environmental factors have been implicated as causing this shift in autogeny. Except for the two past years, the years where blood-feeding was increasing in the southern and Tattnall County populations were also years that experienced below average precipitation (using the base years 1950-1995) (Figure 3). During the past two years, the average precipitation has remained below average at the southern population site in Apalachicola National Forest, and the Tattnall County population has seen a return to near historic levels (Figure 4). Temperatures have remained at historic levels throughout the original blood-feeding trials and the ones conducted in this study. With the Tattnall County population, this result fits well with the expectation that blood-feeding and autogeny are correlated with environmental conditions, such as precipitation. The blood-feeding behavior and autogeny were observed to differ when compared with precipitation levels.

As the precipitation levels fell, the population responded by increasing blood-feeding behavior and shifting towards facultative autogeny.

The olfactory response to the carbon dioxide stimulus is likely to be directly tied to the level of autogeny in mosquito species. Mosquitoes that do not host-seek or blood-feed would not respond to carbon dioxide in the same manner as mosquitoes that are host-seeking. Our results correlate with the anticipated outcome of our comparison between populations. The greater level of blood-feeding behavior in the southern population and the increased sensitivity to carbon dioxide indicate that the southern populations readily seek a blood meal. The Tattnall County population, potentially due to the return to historic environmental conditions and autogeny, was both unwilling to blood-feed and mostly unresponsive to the carbon dioxide stimulus. The vast differences in autogeny among the different populations of *W. smithii* exhibits the effects climate can have on fecundity and blood-feeding behavior. Future research would look at the differences in larval nutrient availability and larval densities in the pitchers of *Sarracenia purpurea*. By comparing the density, nutritional, and environmental components, the underlying cause in the differences in autogeny between disjunct populations. As such, autogeny would not appear to be derived from simple genetic regulation. Autogeny and blood-feeding behavior exists on a spectrum that is influenced by many different factors. Hexamerin storage is one factor that is strongly tied to levels of autogeny. This particular process may be regulated by various hormones, neural pathways, or particular changes in epigenetic structure during development. Further studies are required to determine whether these behaviors are regulated by complex mechanisms or simply the activation of hormone-linked receptors.

[It would be appropriate to speculate on how the plasticity in blood-feeding behavior is regulated, i.e., it doesn't appear that there is a simple genetic determinant of blood-feeding propensity, but rather a "sliding scale", perhaps regulated by some sort of neural or hormonal mechanism that is responsive to levels of hexamerin storage in larvae. It may be as simple as stretch receptor activation that is modulated by brain-related hormones.]

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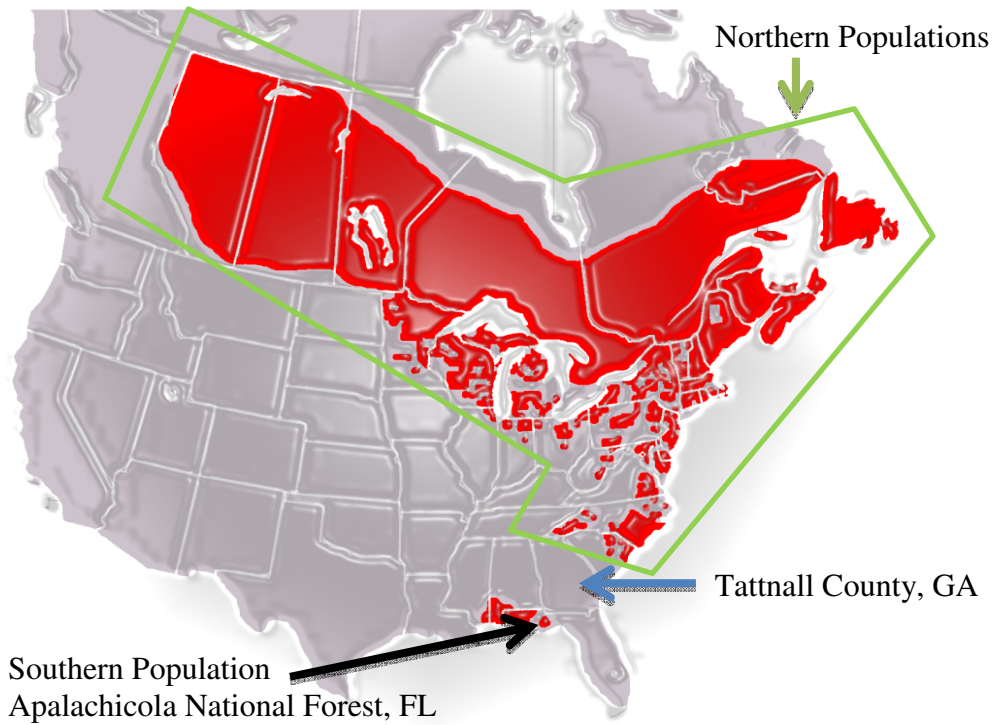


Figure 1: A map outlining the known populations of *Sarracenia purpurea* and *Wyeomyia smithii*. This map shows the regions where different levels of autogeny can be found in *W. smithii*. Each population is labeled, with the northern population (unsampled in this study) enclosed within the border lines.

Table 1. Results of blood-feeding trials\* for post-autogenous adult female *Wyeomyia smithii* from 2004-2011 for populations from Apalachicola, FL and Tattnall Co., GA.

		Ave. % Feeding ( $\pm$ SD) by year				
Population	1998	2004	2007	2008	2011	2015
Apalachicola	+**	ND**	8.0(7.3)	13.8(11.2)	26.0(15.1)	4.8(18.1)
Tattnall	$\pm$	2.3(1.3)	3.8(6.2)	8.6(5.7)	20.8(15.7)	3.3(2.2)

\*6-12 15 minute feeding trials/population, 20-125 female mosquitoes/trial, all blood-fed specimens removed after each trial

\*\*In feeding trials with mosquitoes from Apalachicola, FL, mosquitoes were observed to blood-feed; in trials with Tattnall Co., GA mosquitoes, one female probed but did not feed

\*\*\*not done



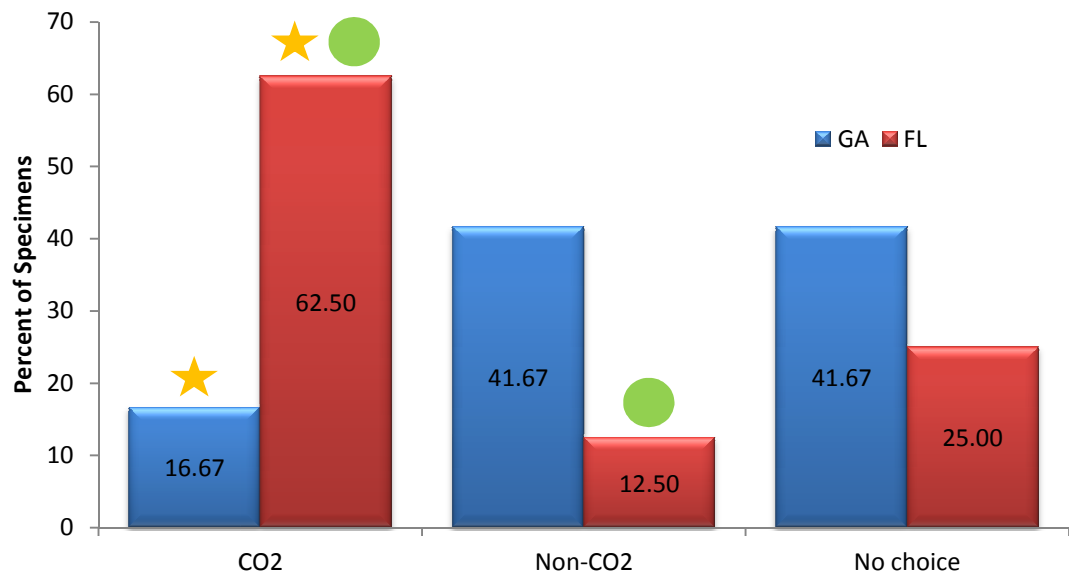


Figure 2: A bar graph showing the percentages for the various choices in olfactometry trials. This figure shows the percent of mosquitoes that chose carbon dioxide (CO<sub>2</sub>), chose a sugar-water control (Non-CO<sub>2</sub>), and did not make a choice (No choice) by population, with the Tattnall County, Georgia (GA) population being represented on the left and the southern population from Apalachicola National Forest (FL) on the right. Stars and circles indicate significant results. The stars indicate a significant difference in the carbon dioxide responses between the GA and FL populations. Circles indicate a significant difference between carbon dioxide and control choices within the FL population.

Table 2: A table showing the results of a pairwise Chi-square analysis of the two choices possible (CO<sub>2</sub> and Control) between the Tattnall County populations (GA) and the Apalachicola National Forest population (FL).

	Chi-Square	df	p-value
GA vs. FL CO <sub>2</sub> Choice	5.74	1	0.0166
Ga vs. FL Control Choice	2.99	1	0.0838
FL CO <sub>2</sub> vs. Control Choice	7.56	1	0.006
GA CO <sub>2</sub> vs. Control Choice	1.87	1	0.1715

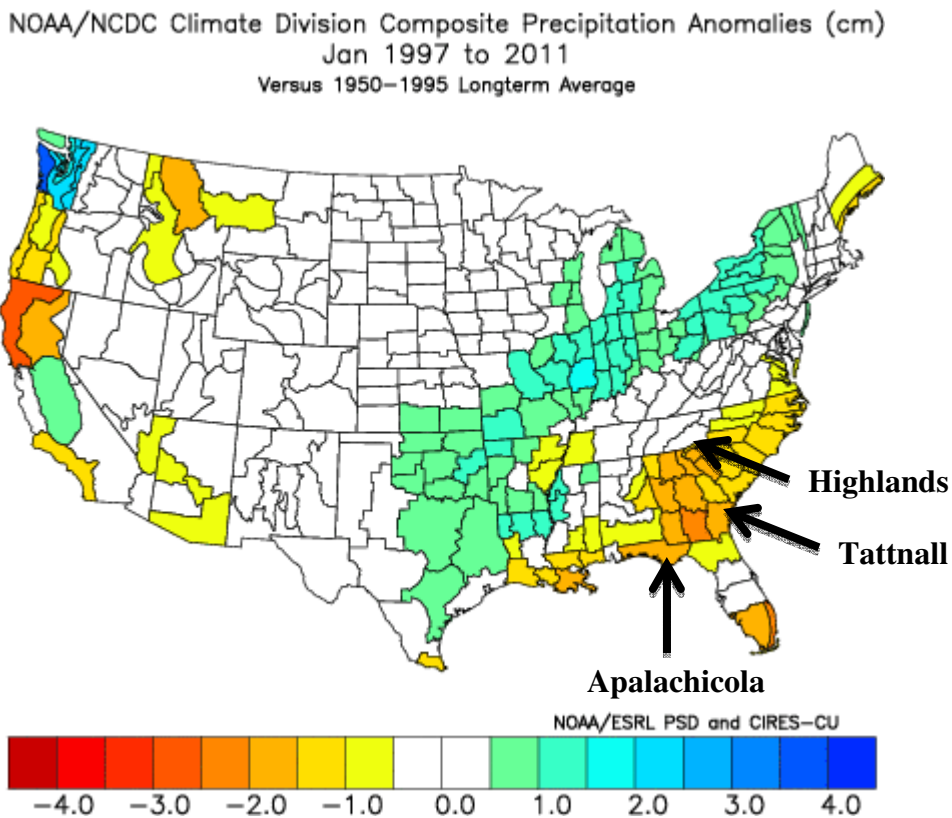


Figure 3: A National Oceanic and Atmospheric Administration map showing the difference in precipitation between base years 1950-1995 and previous study years 1997-2011. This map shows the difference between the historic rainfall and the rainfall that occurred during the years of previous studies conducted, with the three site areas previously studied labeled with arrows. The map clearly indicated a drought during the study period of approximately 2.0 cm in both the Apalachicola (southern) population and the Tattnall County population and about normal rainfall at the Highlands site.

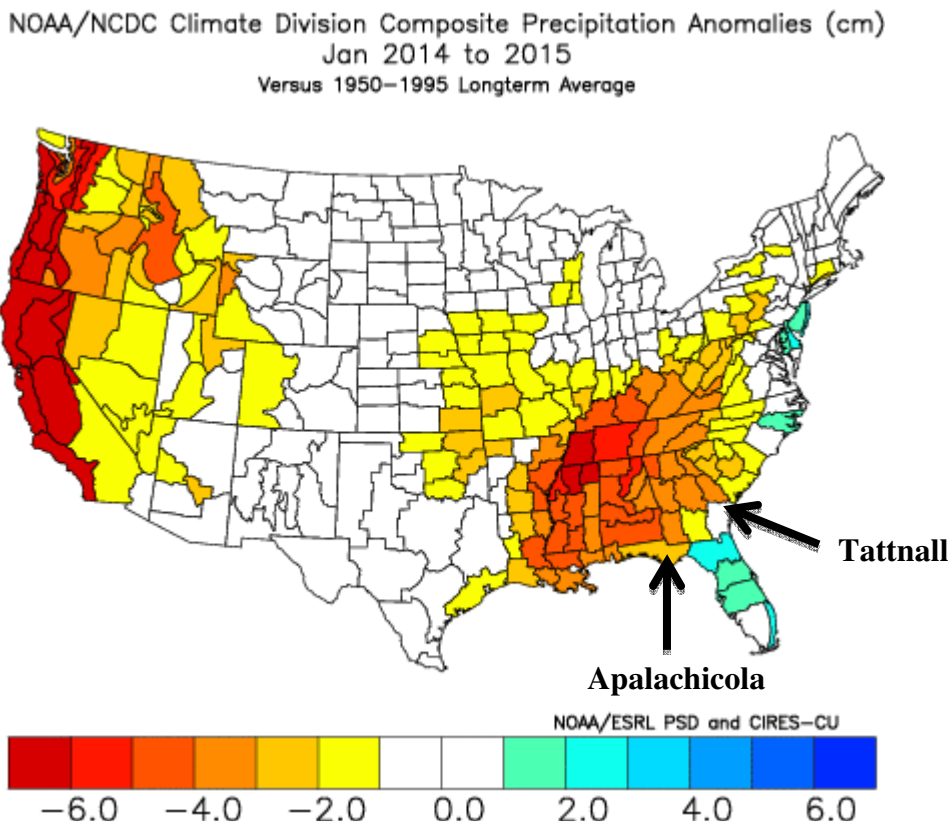


Figure 4: A National Oceanic and Atmospheric Administration map showing the difference in precipitation between base years 1950-1995 and the current study years 2014-2015. This map shows the difference between the historic rainfall and the rainfall that occurred during the years of the current study, with the two site areas labeled with arrows. The map indicated that the Apalachicola (southern) populations are still experiencing lower than average rainfall whereas the precipitation at the Tattnall County population seems to have returned to near the historic levels.