Evolutionary trade-offs and machine learning in Bean Beetles: Behavioral Effects of Juvenile Hormone and the automation of Morphological Measurements

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ABSTRACT

This thesis presents a dual exploration in the study of the bean beetle Callosobruchus maculatus. The first aspect investigates Juvenile Hormone (JH) influence on the behavior and morphology of C. maculatus by manipulating JH levels in adults through abdominal injections. JH is critical for the regulation of both developmental and physiological processes. It is linked to resource allocation trade-offs between wings and gonads during the larval stage. However, its effects on adults are not fully understood. The second aspect pioneers the development of an automated machine learning model for measuring and classifying morphological features using neural networks and computer vision. The findings offer new insights into the behavioral effects of JH and show the potential of automation to revolutionize data collection methodologies in animal behavior. This dual approach not only advances our understanding of evolutionary trade-offs in bean beetles but also showcases the integration of technological advancements in biological research.

DEDICATION

This work is for those who were there to celebrate the wins, the losses, the confusions, and the successes of this journey alongside me.

At its core and beyond, this work is for ***all*** *the Palestinians and the land that I belong to,* ***Palestine****.*

It is for all the Palestinian students, scientists, farmers, writers, thinkers, moms, dads, children, and freedom fighters who were murdered by the Israeli occupation.
I love you.
I wish that you all got to show us your minds, your dreams, your thinking, and your work.
I wish there was no occupation.

To a free and liberated Palestine, from the river to the sea.

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INTRODUCTION

Section 1: Neuroscience and Behavior

1a) Life history theory

Life history theory is a fundamental framework in biology that seeks to understand the allocation of resources among competing life history traits in organisms (Stearns, 1977). Life history traits are influential to organisms' survival, growth, reproduction, and fitness. They include traits such as longevity, growth rate, age and size at maturity, and reproductive patterns (Roff, 1992). At the core of life history theory are trade-offs, which are the inevitable compromises that organisms must undergo when allocating resources to one history trait over another. This unavoidable allocation occurs because resources are finite. Organisms are constantly subjected to environmental and evolutionary pressures that lead to the evolution of allocation strategies for energy, time, and other limited finite resources. These strategies are not the result of conscious decision-making. They are shaped through natural selection to optimize survival and reproductive success. Therefore, investing a resource in one trait reduces the availability of that resource for another trait and vice versa (Barbosa, Rebar, & Greenfield, 2018). For example, some species of birds face trade-offs between clutch size and egg size, balancing the number of offspring with the investment in each offspring's size (Godfray, Partridge & Harvey, 1991).

Even though all species are subject to natural selection to maximize their lifetime reproduction, individuals tend to employ different strategies to achieve that. This leads to trade-offs between different life history traits across species. For instance, species that allocate more energy towards immediate reproductive success face a trade-off between current reproduction and future survival (Johns et al., 2018). Similarly, individuals who invest heavily in offspring development early in life may experience higher risks as they age (Johns et al., 2018). However, trade-offs are not always straightforward. Empirical studies often reveal positive or no correlations between pairs of costly traits where negative correlations were expected (Gascoigne, Uwera Nalukwago & Barbosa, 2022). This inconsistency could be explained by the hierarchical nature of trait investment, as suggested by the tree model of allocation (de Jong, 1993). In this model, different traits occupy different positions in a hierarchical tree, and their covariances are predicted based on their positions. For instance, traits closer to the base of the tree, such as growth and somatic maintenance, are expected to have strong negative covariances, while traits in the upper branches, like reproductive traits, may have weak positive covariances or none at all (Gascoigne, Uwera Nalukwago & Barbosa, 2022; de Jong, 1993). Somatic maintenance includes cellular repair, physiological functions, and resistance to stressors. Thus, in this case, insects face a trade-off between somatic maintenance and reproduction. This trade-off can alter their lifespan, reproductive output, quality of life, and overall fitness. Therefore, individuals that allocate more resources to somatic maintenance may face delayed reproductive maturation with fewer offspring, yet increased longevity (Jacot, Scheuber & Brinkhof, 2004).

Environmental factors, such as larval density in the bean beetle, *Callosobruchus maculatus,* can also influence the pattern of trait covariances by inducing differential resource allocation (Gascoigne, Uwera Nalukwago, & Barbosa, 2022). High larval density, for example, can lead to increased investment in dispersal traits at the expense of reproductive traits, resulting in a trade-off between wing size and gonad size in males (Gascoigne, Uwera Nalukwago, & Barbosa, 2022). Thus, the complexity of trade-offs is often shaped by both environmental factors and individual variability. In the absence of an environmental stressor, trade-offs might not be apparent, as competing traits within an individual may not show distinct negative covariances. Furthermore, the relationship between two traits can differ based on individual differences in acquiring and allocating resources. Mathematical models suggest that a positive covariance between costly traits occurs when individuals significantly vary in resource acquisition but not in allocation, whereas a negative covariance arises when the opposite is true (Noordwijk and de Jong, 1986). Therefore, understanding the complexity of trade-offs is vital to understanding evolutionary biology, adaptations, and mechanisms of fitness optimization (Sih et al., 2010).

Behavior, as a life history trait, is crucial to influencing organisms' fitness and mediating trade-offs. Behaviors including mating, foraging, parental care, and territoriality can play a direct role in organisms' ability to acquire resources, successfully reproduce, or avoid predators (Campbell et al., 2005, Chapter 51 & Stamps, 2007). To further understand how behavior influences trade-offs, researchers have set up various experimental techniques to study it. For example, researchers have tried to investigate growth, reproduction, or survival, which are all fitness-related traits, through manipulating certain behaviors.

These manipulations can be understood through phenotypic correlations, experimental manipulations, genetic correlations, and correlated responses to selection (Bolund, 2020). Phenotypic correlations involve observing the relationship between different traits within a population. For example, researchers might study the correlation between foraging behavior and reproductive success in a population of birds. This approach can help identify potential trade-offs between different behaviors. Alternatively, Farina and Gil experimentally manipulated factors that alter foraging behavior through manipulating food resources in honeybee's environment; allowing them to measure life history traits, showcasing that experimental manipulations involve directly altering a behavior and observing the effects on other traits (Gil & Farina, 2002). Whereas genetic correlations involve studying the genetic basis of different traits and how they are related. For example, researchers like Moore et al., investigated the genetic correlation between aggressive behavior and territoriality in a population of Tuatara (Moore, Daugherty, & Nelson, 2009). This approach can help identify genetic factors that influence multiple behaviors. Further, researchers comparatively examined how behavioral variation correlates with adaptations and differences in life history traits and trade-offs among different species, taxa, and populations (Leroi, Rose, & Lauder, 1994). In other words, Leroi et al. (1994) correlational approach can help identify how selection on one behavior can influence other traits.

Each one of those techniques has its advantages and disadvantages. Phenotypic correlations, for example, are relatively easy to measure in natural populations and can provide insights into potential trade-offs between different traits. However, they do not imply causation and may be confounded by environmental factors. Therefore, if the aim is to imply causation, the use of experimental techniques is advised. Experimental manipulations can establish causality because they directly manipulate one trait and observe the effects on others. Nonetheless, they are limited by the difficulty of replicating natural conditions and can raise ethical concerns at times. Additionally, genetic correlations are useful for understanding the heritability of life history traits and their relation to other traits, but they require knowledge of the genetic architecture and may not capture the full complexity of behaviors influenced by multiple genes and environmental factors. Lastly, correlated responses to selection can illustrate the evolutionary consequences of selection on one trait for other traits and help in understanding how traits evolve together over time, but they require long-term studies and can be challenging to interpret in complex ecological contexts.

The ability to understand and examine behavior as a life history trait equips us with valuable insights into the evolutionary and adaptive strategies that organisms utilize to cope with ecological and environmental challenges in order to optimize their fitness. However, even though there has been an extensive amount of research into life history theory, there are still limitations in our understanding of behavior as a life history trait (Sih et al., 2010). We need to further integrate behavioral studies with physiological and morphological exploration of life history traits. By doing so, we can fill these gaps in our knowledge and gain deeper understanding of how behaviors contribute to fitness, trade-offs, and evolutionary adaptations. Besides, we could further understand the mechanisms influencing behavior and impacting trade-offs.

Insects are a particularly interesting group for studying behaviors within the context of evolution and life history traits. They exhibit diverse behavioral patterns, reproductive strategies, and unique interactions with their environments (Mousseau & Fox, 1998). They also face lots of ecological challenges, including predation, competition for resources, and environmental variability, that force them to constantly encounter trade-offs (Sih, Bell, & Johnson, 2004). Furthermore, insects make a great study organism for exploring complex life history traits and trade-offs. Besides the ease of their laboratory maintenance, they live relatively short generation times and in large population sizes. Thus, we are able to study neural mechanisms underlying their behavior through various available techniques, making them ideal for exploring hormonal influences on behavior, especially since they rely on hormones to regulate various aspects of their behavior.

1b) Hormones and behavior

In the context of life history theory and trade-offs, understanding the role of hormones in mediating behavior provides insights into the mechanisms underlying evolutionary adaptations and fitness-related trade-offs. Hormones act as chemical messengers that regulate neural processes and control the expression of life history traits and behaviors such as foraging, mating, reproduction, social interactions, and aggression (Nelson, 2024; Karigo & Deutsch, 2022; Garland, Zhao, & Saltzman, 2016). They can influence insects' adaptive responses by integrating both internal physiological states with external environmental cues. Therefore, measuring behavior allows us to deepen our understanding of the hormonal influence that is regulating certain behaviors. Even though this thesis focuses on manipulating juvenile hormones, various hormones affect insect behavior.

Ecdysteroids are insect hormones that are known for regulating developmental processes like metamorphosis and molting (Truman & Riddiford, 2002). They were found to be involved in insect adult behaviors as well (Mirth & Riddiford, 2007). Variations in their levels have been linked to changes in multiple aspects of insects' behavior. For example, researchers have found that fluctuations in vitellogenin, which is a yolk precursor protein impacted by ecdysteroid levels, trigger changes in foraging and labor behavior in honebees, *Apis mellifera*, impacting their ability to locate, acquire, and utilize food resources differently. (Amdam et al., 2003) Moreover, ecdysteroids levels influence reproductive activities, which influences mate-seeking behaviors, courtship rituals, copulation, and oviposition behaviors that are vital for reproductive success (Rauschenbach et al., 2000). Additionally, ecdysteroids have been implicated in regulating circadian rhythms and sleep patterns in some insect species such as adult fruitflies, *Drosophila melanogaster,* affecting their courtship memory (Ishimoto, Sakai, & Kitamoto, 2009). These regulations affect the timing and duration of locomotor activity, rest periods, and daily behavioral rhythms, which are all vital for optimizing resource allocation. Therefore, ecdysteriods play a significant role in insects physiology, behavior, and life history traits.

Similarly, neuropeptides and neurohormones impact behavior through modulation of the nervous system. Neuropeptides like octopamine serotonin play essential roles in regulating locomotor activities and feeding behaviors in *Caenorhabditis elegans* (Flavell et al. 2013). Additionally, Andrews et al. (2014) found that octopamine modulates social behaviors, including aggression and courtship in Drosophila melanogaster. Furthermore, research on multiple organisms including honeybee and Drosophila melanogaster has found that octopamine is insects' equivalent of adrenaline and can influence arousal, motivation, and stress responses (Bobrovskikh & Gruntenko, 2023; Even, Devaud, & Barron, 2012). Thus, fluctuations in neuropeptides levels influence behaviors related to resource acquisition and predator avoidance. Likewise, neurohormones such as corazonin have been implicated in regulating behaviors related to insect stress responses and aggression (Zhao et al., 2010; Khan et al., 2021). It functions similarly to the mammalian Gonadotrophin Releasing Hormone (GnRH), indicating its significant role in development, internal states, and behavioral decision-making. Corazonin regulates systemic growth, food intake, stress responses, and homeostasis by interacting with short Neuropeptide F (sNPF) and the steroid hormone ecdysone (Khan et al., 2021). Corazonin neurons also have a sex-dependent lifespan differences under various stresses in Drosophila melanogaster (Zhao et al., 2010) Comparably, insulin-like peptides and adipokinetic hormones (AKH) influence feeding behaviors, metabolism, and energy balance in response to nutritional cues and environmental stressors (Koyama et al., 2020). They act in a manner analogous to insulin in mammals, with effects on the insulin signaling pathway being central to stress resistance, lifespan, and metabolic homeostasis (Zhang & Liu, 2014; Koyama et al., 2020)

Lastly, insects' sex hormones, which include ecdysteriods and juvenile hormones, play an essential role in regulating reproductive behaviors. They modulate sex-specific behaviors and sexual differentiation in insects as well as various other life-history traits (Pan, Connacher & O'Connor, 2021). JH is the most well-known hormone in insects due to its involvement in many parts of their lives. It has been shown that an increase in JH levels is accompanied by an increase in mating behaviors and reproductive success in D. melanogaster and bedbugs (Flatt et al., 2005; Reiff et al., 2015; Gujar & Palli 2016). Similarly, an increase in JH titer is often correlated with increased aggression (Pandey, Motro & Bloch, 2020). These findings highlight the importance of further investigating JH due to their complex and diverse involvement in insects' life cycle from early development to adulthood.

1c) Juvenile Hormones

Juvenile hormones are a group of hormones that highly contribute to the development of insects, particularly regarding insects' sexual growth and maturation as well as morphology (Koeppe et al., 1985). They have been well-documented in their involvement in modulating the development of trait polymorphism and sexual dimorphism (Zera, 2004; Guerra, 2011). Trait polymorphism refers to the occurrence of two or more distinct traits, such as color, size, or shape, within a population of the same species. Usually, polymorphism allows a species to better adapt to the environment. For example, many butterflies' populations have polymorphic wing patterns, allowing them to better blend into their respective environments (Wallbank et al., 2016). Whereas sexual dimorphism refers to the sex-based differences in appearance within the same species. These differences are beyond their sexual organs. They can include variations in size, color, or the presence of certain phenotypic features. For example, peacocks tail feathers are sex-dependent where male peacocks have colorful tail feathers while the female peacocks have dim subdued feather coloration (Petrie, Halliday & Sanders, 1991).

JH are insect acyclic sesquiterpenoids produced by the corpora allata (CA), which is a tiny factory-like organ near the insects' brain that produces important hormones. The surgical removal of corpora allata eliminates the source of JH secretion (Yamamoto, R. et al., 2013). Acyclic sesquiterpenoids are a subgroup of complex organic chemicals called isoprene, which act as building blocks. Isoprenes come together in a particular way to create juvenile hormones. However, JH biosynthesis is controlled by neuroendocrine and neuronal factors (Shinoda, 2016). This means that the production of JH is complex and controlled by various conditions, which matches its role. The primary functions of JH are linked to molting, wing development, and sexual maturation (Iwanaga, & Tojo, 1986; Koeppe et al., 1985). For instance, elevated levels of JH in caterpillars are associated with larval molting, whereas low JH levels signal the initiation of pupation, which leads to adult formation through metamorphosis (Yamamoto et al., 2013; Kayukawa et al., 2017). Likewise, wing development is influenced by JH in various degrees. For example, JH promotes Br–C expression, which in turn promotes wing growth and development in cockroaches (Fernandez-Nicolas et al., 2022). Similarly, Zhang et al (2020) found that JH regulates flight capacity and migration within a sensitive period in armyworm, indicating the importance of time and the sensitivity of development to time windows. Lastly, researchers have noted that JH levels fluctuate to reflect insects' reproductive organ development. For example, JH levels increase at the onset of sexual maturity, ensuring that reproduction occurs only when individuals are physically capable of it (Robinson & Vargo, 1997).

During the pre-emergence stages of insects, JHs are involved in coordinating the development and differentiation of various tissues and organs (Truman et al., 2024). They also regulate multiple essential processes, such as larval growth, metamorphosis, and the formation of adult structures and features. For example, JH delays metamorphosis until larvae reach an appropriate stage (Smykal et al., 2014). They also prevent ecdysone-induced changes in gene expression, which are essential for metamorphosis (Gilbert, 2000). This is essential for ensuring the emergence of the right structures. Thus, previous research has shown the importance of JH for shaping morphology and developmental trajectories during those pre-emergence stages.

JHs continue playing an essential role in insects during the post-emergence stages. They are key to modulating biological processes such as ovarian maturation, behavior, caste determination, diapause, stress response, and life span during adulthood (Rahman et al., 2017). Particularly since JHs are secreted in different concentrations and levels by the corpus allatus throughout most of the insects' life cycle. For example, blocking JH action in adult female Locusta migratoria eliminates the production of the major yolk protein (vitellogenesis) or induce vitellogenesis with higher JH (Grozinger et al., 2014). Whereas in honeybees, studies have shown that JH acts as a behavioral peacemaker; as it regulates the speed in which worker bees grow, transition from nest activities to foraging (Robinson & Vargo, 1997). These findings indicate JH's role as a gonadotropin hormone with a variety of essential roles that are time dependent (Koeppe et al. 1985). For instance, early manipulation of JH during the adult stage has induced sexually dimorphic effects on the behavior of Drosophila melanogaster's mature adults (Argue, K. J. et al., 2013). The sexually dimorphic role for JH in the modulation of insects' behaviors post-emergence seems to be contingent on the age of adults (Argue, K. J. et al., 2013).

Guo, W. and colleagues (2020) explored the influence of JH on the behavior of locusts, which are a group of grasshopper species. Interestingly, they found that JH induced a complete behavioral shift from attraction to repulsion and vice versa. Their study suggests the involvement of JH in mediating a cascade of internal physiological processes that control how they act (Guo, W. et al., 2020). JH can do so by binding to specific receptors on target tissues and modulating gene expression. This modulation ultimately influences the development of traits mentioned earlier, like polymorphism, sexual dimorphism, and behavior.

Therefore, juvenile hormones and life history traits as well as trade-offs are intimately linked. They influence the direction of resource allocation. Thus, directing resource investment towards reproduction, survival, maturation, or growth. For example, in resource-limited conditions, JH would prioritize survival over reproduction, which will lead to a delay in maturation, reduction in fecundity, or an increased investment in somatic maintenance (Dao-Wei Z. et al., 2019). The opposite would be expected to happen with an abundance of resources, where JH would favor allocating more resources for reproduction and sexual maturity in insects. As highlighted, juvenile hormones mediate many trade-offs that insects face under different conditions.

1d) Development

It is evident that insects undergo many trade-offs. However, some trade-offs are more well-documented than others. Nonetheless, many trade-offs occur during the developmental stages. For instance, insects must balance investment in growth and body size with investment in reproductive processes and structures (Breiner, Whalen, & Worthington, 2022). Individuals that allocate more resources to growth may delay sexual maturation and reproductive investment, potentially compromising their immediate reproductive success and vice versa (Barbosa, Rebar & Greenfield, 2018).

A well-documented developmental trade-off is between wing development and reproduction. Researchers suggest that JH mediates trade-offs between investment in wing development and reproductive efforts (Zera, 2004). This trade-off between wings and reproduction is a common phenomenon observed in insects, where resources allocated to wing development may significantly impact the resources available for reproduction, influencing an insect's fitness and survival in its environment (Zera, 2004; Contreras-Garduño et al., 2011). The trade-off between investing in wings versus reproduction is not just a matter of resource distribution. It also involves hormonal regulation, with JH being at the forefront of this regulatory mechanism. Therefore, explaining the mechanisms underlying the effect of hormones on development and trade-offs would allow us to gain a deeper understanding of how insects adapt to their environment. Especially because JH has a pivotal role in mediating this trade-off between wings and reproduction. This trade-off is evident in wing polymorphic insects. For example, Zera & Zhang (1995) showed that a reduced activity of juvenile hormone esterase, which is linked to a lower JH degradation, correlates with the development of short-winged morphs in the Jamaican field crickets. In other words, this means that higher JH levels are correlated with the development of short-winged cricket morphs. Alternatively, aphids provide another interesting exploration pathway. Aphids are small yet ecologically significant pests. They exhibit an evolutionary adaptation known as wing dimorphism, where individuals within the same species can develop to be either winged or wingless morph. This adaptation occurs due to environmental challenges and pressures such as overcrowding and resource scarcity. Each one of the two morphs has its advantages, yet it comes at a cost. For example, winged morphs can disperse to new locations when conditions become unfavorable, allowing the species to spread and find new resources. However, the dispersal comes at the cost of reproduction. On the other hand, the wingless morphs are more common when conditions are stable, focusing on rapid reproduction without the energy cost of developing wings (Blackman & Eastop, 2000). This evolutionary change represents a strategic adaptation that ensures the survival of aphids (Ogawa & Miura, 2014). Here, too, JH mediates the trade-off between wing development and reproduction. Researchers have found that JH levels significantly affect the development of winged versus wingless morphs in aphids (Braendle et al., 2006). JH acts as a mediator between environmental and developmental cues (Brisson, 2010; Braendle et al., 2006). In particular, high levels of JH are associated with the promotion of the wingless morph, encouraging rapid reproduction in favorable conditions (Braendle et al., 2006). Conversely, low JH levels can trigger the development of winged morphs, preparing individuals for dispersal in response to environmental stressors (Braendle et al., 2006). Here, we see JH playing a pivotal role in developing both physical and mechanistic responses to environmental stressors and trade-offs.

1e) Bean Beetles

Bean beetles, Callosobruchus maculatus, were used to explore the aims of this research. These beetles typically have a reddish-brown to black coloration and measure around 2-4 millimeters in length as adults. Callosobruchus maculatus is a species that has been key to studying behavior (Gascoigne et al., 2021;), morphology (Gascoigne et al., 2021;), molecular and biochemical (Zelaya et al., 2020; Berasategui et al., 2021), as well as life-history traits and trade-offs (Gascoigne et al., 2021; Schade & Vamosi, 2012). They make a great match for understanding the hormonal influence on behavior for various reasons. In addition to having been used intensively, the bean beetle's life cycle is key to understanding the effect of hormonal manipulations. They undergo a complete life cycle, which consists of egg, larva, pupa, and adult beetle. They take around 4 weeks to emerge out of their beans and don't require any food. Additionally, adult bean beetles experience short adulthood, lasting only 10 days, with 1-2 days to reach sexual maturity post-emergence. Therefore, these characteristics of C. maculatus life cycle make it suitable for studying hormonal manipulation during early adulthood.

Nonetheless, previous research is limited in outlining the role of JH during adulthood. This gap in knowledge presents an opportunity for further investigation into JH functions and mechanisms of action, particularly during adulthood. Thus, this study aims to explore the influence of JH during adulthood on morphology and behavior in bean beetles. Therefore, I intend to expand our understanding of the link between hormones, behavioral, and morphological traits during adulthood. Additionally, this project is an attempt to expand our understanding of the role that time and development periods have on behavior and morphology.

1f) Research, Aim, and Hypothesis

Before exploring the study's aim, we generated a hypothesis to predict the research findings. I hypothesize that in the adult stage, JH mediates behavioral traits in male and female C. maculatus with little to no impact on morphological traits. Specifically, higher JH titter will intensify mating behaviors in male and female bean beetles. Previous research done in the lab has started exploring the effects of JH on C. maculatus resource allocation on their behavioral and morphological traits. That research focused on manipulating JH during the larval stage and later measuring adult traits. Thus, allowing us to have a better understanding of the underlying hormonal mechanisms mediating the hierarchical trade-offs in morphology and behavior pre-adulthood. Later it showed that morphology, but not behavior, was influenced by the hormonal changes. Therefore, the previous JH research imposes a need to investigate the role of JH during adulthood through looking at both morphology and behavior.

Section 2: Data Science

Time is scarce. Driven by that thought and the need to optimize time, the idea for this project was born. More specifically, during my time at the Barbosa Lab, I lived and witnessed hours pass by as my lab mates and I attempted to classify and measure the various morphological aspects of the dissected beetle. This lengthy process becomes time-consuming when dealing with hundreds of samples. Therefore, the main computational problem that my thesis explores is finding an automated measurement machine learning model and a classification model for the various classes using computer vision.

2a) History of Machine Learning Evolution

The journey of machine learning (ML) began in the mid-20th century, rooted in the desire to create computational models that can adapt and learn from data. The first explorations in the field were influenced by Warren McCulloch and Walter Pitts' work on cybernetics and the idea of neural networks in 1943 (Piccinini, 2020). These models were inspired by the understanding of biological processes and aimed to replicate the way human brains operate (Shepherd, 2010).

In the 1950s, Alan Turing proposed the concept of a machine that could learn and evolve (Turing, 1950). This concept was the base of the "Turing Test", which became a criterion for intelligence (Turing, 1950). This era also saw the development of the Perceptron by Frank Rosenblatt in 1957, which is an early neural network that could perform simple classification tasks (Copeland, 2024). However, the excitement was tempered by the realization in the 1960s and 1970s that these early models had limitations, particularly in their ability to solve non-linear problems or learn complex patterns, which killed the interest in ML and AI (Haigh, 2024; history, n.d.). Nonetheless, the 1980s witnessed a revival of interest in ML due to the introduction of new algorithms and models, including decision trees, Support Vector Machines (SVM), and backpropagation for neural networks (Polson & Sokolov, 2020; Rumelhart, Hinton, & Williams, 1986; Cortes & Vapnik, 1995). These developments, alongside increased computational power and the accumulation of larger datasets, set the stage for significant advancements and the regeneration of people's interests and hopes.

Following the 80s developments, the 1990s and 2000s witnessed the consolidation of ML as a critical component of artificial intelligence, with the introduction of deep learning and reinforcement learning (Haigh, 2024; Li, 2018). This was particularly driven by the success of deep learning models, especially Convolutional Neural Networks (CNNs), in image recognition tasks, highlighting the potential of ML to address increasingly complex problems and non-linear tasks.

2b) Computational Review of the Evolution of Machine Learning

Here, I want to explore the evolution of machine learning through a mathematical lens. Especially since the history of machine learning is tied to increasing complexity in mathematical models and computational techniques.

Perceptron (1957): The Perceptron model is considered one of the earliest neural network architectures. It computes a weighted sum of its input features and applies a step function to determine the output class. Mathematically, the Perceptron's decision function for an input vector x can be represented as:

$$y = f(w \cdot x + b)$$

where **w** denotes the weight vector, **x** represents the input feature vector, and ***b*** is the bias. This model laid the foundational computational framework for later neural networks, illustrating the potential of weight-based computation for classification tasks (Li, 2018).

Backpropagation (1980s): The discovery of the backpropagation algorithm marked a significant advancement in training multi-layer neural networks and exploring non-linear relationships, which revived the interest in ML. This algorithm uses the chain rule of calculus to compute gradients efficiently for each layer in a network. This process is essential for adjusting the network's weights during training. Specifically, backpropagation calculates the gradient of the loss function with respect to each weight by the chain rule, enabling a systematic reduction in error through gradient descent optimization. The computational expression for updating a weight wij in the network is given by:

$$w_{ij}(new) = w_{ij}(old) - \eta(\partial L/\partial w_{ij})$$

where **η** *is the learning rate,* ***L*** is the loss function, and ($\partial \mathbf{L}/\partial \mathbf{w}_{ij}$) represents the partial derivative of the loss with respect to the weight $\mathbf{w}_{ij}$. This discovery laid the groundwork for the development of deep learning (Sukhbaatar, Szlam, & Fergus, 2016; Li, 2018).

Support Vector Machines (1990s): SVMs introduce a different approach to classification, focusing on the construction of an optimal hyperplane that maximizes the margin between different classes. The primary goal of an SVM is to determine the optimal hyperplane that separates classes in a feature space. The optimal hyperplane is the one that has the maximum margin, which is the largest distance between the hyperplane and the nearest points of any class (known as support vectors). The optimization problem can be formalized as follows:

$$\text{Min}\,\frac{1}{2}\|w\|^2 \text{ Subject to } y_i(\mathrm{w}\cdot x_i+\mathrm{b})\geq 1 \text{ for all } i$$

where $\mathbf{x}_i$ are the input vectors, $\mathbf{y}_i$ are the class labels, **w** is the normal vector to the hyperplane, ***b*** is the bias, and **||w||** is the Euclidean norm of the vector w. This formulation leads to a convex optimization problem that ensures a global minimum allowing SVMs to handle linear and non-linear classification (Scholkopff, & Smola, 2001; Li, 2018).

Convolutional Neural Networks (CNNs): CNNs, a class of deep neural networks, are specially designed for processing data with a grid-like topology, such as images. A CNN autonomously learns hierarchical patterns in data through convolutional layers, which apply filters to capture spatial dependencies in input data. The operation within a convolutional layer for a filter k can be mathematically described as:

$$S(i,j) = (I\cdot K)(i,j) = \sum_{m}\sum_{n} I(m,n)K(i-m,j-n)$$

Where **S(i,j)** represents the output of the convolution at point (**i,j), *I*** is the input image, **K** is the kernel (filter) applied to the image, and **S** is the feature map produced by the convolution, m is the row indices, and n is the column indices. This algorithm iterates over the entire image and applies the kernel in order to produce the feature map. This formula, combined with pooling layers and fully connected layers, enables CNNs to learn complex patterns in data, from basic edges to intricate object features, which makes them exceptionally capable and suitable for complex image recognition tasks (Zagoruyko & Komodakis, 2017; Li, 2018; Alzubaidi et al., 2021).

2c) History of Computer Vision and Classification

Classification algorithms are at the heart of many machine learning and computer vision tasks. At its core, computer vision seeks to replicate the human visual system, allowing computers to identify, process, and interpret visual data (Blei and Smyth, 2017; Provost and Fawcett, 2013). The beginning of this field can be traced back to the 1960s with the emergence of Artificial Intelligence and the work of Larry Roberts (Verdict, 2020). Larry Roberts in 1963 worked on reconstructing 3D images from 2D images, which marked the initial steps toward enabling machines to interpret visual data similarly to how humans do (Verdict, 2020).

However, computer vision's early years were focused on basic tasks such as pattern recognition and simple object detection. These tasks were approached with rule-based algorithms that processed images as multi-dimensional arrays of pixel intensities. Techniques like filtering, thresholding, and edge detection were developed to manipulate these pixel values for extracting meaningful information, which laid the groundwork for the present time's more complex image understanding.

Therefore, during the 1970s and 1980s research efforts focused on the development of foundational algorithms for more advanced image processing tasks. These included complex edge detection, feature extraction, and understanding the motion structure, crucial for recognizing shapes and objects within images. For example, in the 1970s, scientists started developing optical character recognition technology (Schantz, 1982). This technology allowed computers to be able to recognize printed text. Alternatively, in the 1980s, neuro and computer scientists like David Murr and Kunihiko Fukushima integrated their intersectional work to produce pivotal image and visual processing models, edge-detecting algorithms, and neural networks' convolutional layers (Russell, and Norvig, 2009; Li, 2018). Thus, this period was marked by an exploration of how to accurately represent and categorize visual information, and integrating machine learning techniques, which allowed scientists to move from rule-based processing to models that could learn from data.

2d) Computational Review Main Computer Vision Concepts

Here, I will explore the main concepts of computer vision through a mathematical lens.

Edge Detection: Mathematically, edge detection can be seen through the lens of gradient calculation. The gradient of an image, represented as $\nabla I(x,y)$, measures the change in intensity across the image. A popular method for finding edges is the Sobel operator, which approximates gradients by convolving the image with a pair of 3x3 kernels, one estimating the gradient in the x-direction (G_x) and the other in the y-direction (G_y).

The magnitude of the gradient is given by:

$$|\nabla I(x,y)| = \sqrt{(Gx^2+Gy^2)}$$

(BenHajyoussef, & Saidani, 2024)

Feature Extraction: Feature Extraction can identify and isolate meaningful attributes or characteristics within the image data. It translates raw image data into a format or set of features suitable for classification algorithms (Vega-Rodriguez, 2004). A common framework for feature extraction is Principal Component Analysis (PCA), which seeks to reduce the dimensionality of the data while preserving as much variance as possible (Banerji, Zunker, & Sinha, 2020). It is essentially a process of simplifying complex data. Imagine having a dataset with numerous variables, and some of these variables share similar information. PCA helps find a more straightforward way to express this data without losing much of its valuable information. It identifies the main patterns in the data (directions where there is the most variance) and redefines the dataset in terms of these patterns (Banerji, Zunker, & Sinha, 2020). This results in a simplified version of the data, where the most important trends are highlighted, and the less informative, redundant aspects are minimized. PCA involves calculating the eigenvectors (non-zero vectors that do not change direction) and eigenvalues (magnitude of the stretch) of the data's covariance matrix, then selecting those that correspond to the largest eigenvalues, and using them to transform the original data into a new space with reduced dimensionality (Banerji, Sinha & Liu, 2012; Banerji, Zunker, & Sinha, 2020). Alternatively, a Histogram of Oriented Gradients (HOG) is used to obtain a representation of the image's shape and texture (Banerji, Sinha & Liu, 2012). It involves calculating and binning gradient directions across localized regions of an image, which encapsulates the structure within a feature vector (Banerji, Sinha & Liu, 2012). In particular, HOG calculates each pixel's gradient's magnitude and direction and then these gradients are accumulated in a histogram over specific image regions (Banerji, Sinha & Liu, 2012).

$$\theta(x\,,\,y) = \arctan\left(\frac{G_x}{G_y}\right),\ |\nabla I(x,y)| = \sqrt{(G_X^2+G_y^2)}$$

In other words, the image is divided into small, connected regions, called cells, and for each cell, a histogram of gradient directions or edge orientations is compiled. The concatenation of these histograms then forms the feature descriptor.

2e) Feature Descriptor Techniques

In the field of image analysis, feature descriptors play a crucial role in transforming raw data into a more compact and expressive representation for further processing. In this study, four main types of feature descriptors were used to extract meaningful information from images of bean beetles: Local Binary Patterns (LBP), Histogram of Oriented Gradients (HOG), Contour-Based features, and Color Histograms. Each one of these descriptors captures unique characteristics of the images, which are pivotal for the subsequent classification and analysis tasks.

Local Binary Patterns (LBP) is a texture descriptor that is used extensively in image analysis due to its robustness and computational efficiency. LBP operates by comparing each pixel with its surrounding neighbors, assigning a binary code that results from the comparison. These binary codes are then compiled into a histogram, which serves as the final feature descriptor. LBP is particularly effective in capturing fine textural patterns, making it well-suited for differentiating subtle variations in texture that distinguish various morphological traits.

The Histogram of Oriented Gradients (HOG) descriptor is primarily used for object detection and is highly effective at capturing edge and gradient structures. HOG works by dividing the image into small, interconnected regions known as cells, where for each cell, a histogram of gradient directions or edge orientations is compiled. The complied histograms get normalized over larger blocks of cells to improve accuracy and provide resistance to illumination variations. The combination of these histograms forms the feature descriptor.

Contour-Based features focus on capturing the shape and silhouette of objects within an image, which is achieved by analyzing the boundaries or contours of the objects. This technique is particularly useful for distinguishing objects based on their geometric and spatial characteristics. Notably, these features discard the information about the color distribution in the images. Therefore, the extracted features are a full representation of the edges and shape of the object rather than the combination of that with its colors.

Lastly, Color Histograms, on the contrary, are used to represent the distribution of colors within an image. By measuring the intensity of different color bins across the image, this descriptor provides a robust perception of color distribution which can be crucial for classification tasks where color is a distinguishing feature.

2f) Rationale, Hypothesis, and Aim

Based on the literature review and a thorough examination of the dataset, data types, and project needs, I hypothesize that using Support Vector Machine models would be able to classify the classes well. However, I anticipate challenges with feature extractions due to the nature of my images. Therefore, I predict a need for various feature extraction techniques in order to maximize classification accuracy. Further, I believe that the use of pre-trained machine learning models would be useful for further training a model to measure the length of the morphological trait. Therefore, I hypothesize that an intersection between manual and automated processes would lead to the best outcome when it comes to building a model with the highest measurement accuracy and best reliability.

METHODS AND MATERIALS

Beetles Population

The study used individuals from a laboratory population at Lake Forest College. The population started in 2018 from hundreds of individuals from the strain of Callosobruchus maculatus. It is kept in several sealed 4-L plastic containers with mesh tops for ventilation and adzuki beans. We maintained this population under a breeding protocol to avoid in-breeding of the population and any confounding variables. The protocol included daily and weekly check-ins, the addition of new adzuki beans when needed, and clearing any dead or damaged beans using featherweight forceps.

Egg lay and density Assignment

To generate individuals for our study, we reared individuals from egg to adulthood under controlled larval density in clear 56-well plates with lids. We did this by adding a single adzuki bean and a single female to each well of the 56-well plate, for a total of 14 plates. Therefore, the females' sexual history was unknown; however, they had access to males in the general population. Thus, the likelihood of having reached sexual maturity and successfully mated, at least once, was high. We allowed the females to lay eggs on the beans for a week. Then, to ensure the individuals fall into the medium density group, we removed any extra eggs by scraping the eggs off with the tip of a pair of featherweight forceps if a bean had more eggs than the needed range of 7-9 eggs per bean. Alternatively, any bean that had fewer eggs than the needed range was kept in its well as is. The plates were stored in an incubator (Power Scientific environmental chamber DS27SD) at 27°C under a 12L:12D photoperiod until the completion of the beetles' emergencies from their beans. Adult beetles started emerging between 4 and 6 weeks after oviposition. Subsequently, all plates were checked multiple times daily for any emergencies starting at the 4-week mark. Any adult that merged was identified using a unique ID based on the plate number and well ID. For example, 1-F8 refers to the beetle from plate 1 that was recorded from well F8; wells are represented through a matrix that is vertically and horizontally labeled: A-F and 1-8, respectively.

Beetle Injections

We manipulated the beetles by randomly dividing them into different injection groups: Methoprene, Precocene, and Acetone. We injected each emerging adult on the day of their emergence with 0.2 µL of either one of those treatments using a Hamilton Syringe. All injections were intersegmental membrane (abdomen) injections. Beetles had a 24-hour period to recover where they were kept isolated in their wells post-injection.

Behavioral Assay

To conduct the behavioral assays, experimental beetles were IDed, sexed, injected, and individually isolated in 1.5 mL centrifuge tubes on the day of their emergence. We only used one beetle per bean to reduce the biases in our data collection process. Non-experimental beetles were sexed and individually isolated in 1.5 mL centrifuge tubes on the day of their emergence. All beetles were massed prior to mating them, which happened 24-48 hours after their emergence to ensure their sexual maturity (Beck and Blumer, 2014) and recovery if injected. Mating events occurred in a petri dish with a lid where two beetles, experimental and non-experimental, of opposite sexes were recorded for the entirety of their mating duration. For all mating recordings, females were added first to have an accumulation period of 1-2 minutes to reduce handling and environmental effects.

Dissections and Morphological Imaging/Measurements

After the behavioral assay, females were individually left with 10 beans in clear plastic cups with flat lids for 72 hours (3 days). When completed, females were put into 1.5 mL centrifuge tubes, and 70% ethanol was added to prepare for dissection. Both males and females were dissected using FST fine forceps with mirror finish, microscope, and Pyrex 9-well dissection plates. Appendages were mounted, to prepare for imaging, on either raised or non-raised coverslips based on whether their tissue is compressible or not. All appendages were mounted in DHMF, a mixture of 30g DHMF resin with 30 mL 70% ethanol. Table 1 references the details.

Mounted specimens were imaged using Lecia stereoscope, digital camera, and imagining software application. These imaged appendages were measured using ImageJ. Table 2 references the details of our operationalized measurement schema.

Table 1

Sex	Raised Coverslip	Non-Raised Coverslip
Male	Leg	Wing
	Elytron	Antennae
	Testes	
Female	Leg	Ovariole
		Wing
	Elytron	Antennae

Table 2

Morphological Traits and Their Measurements

This table details a list of various morphological traits, their images, and detailed descriptions of how they were measured on ImageJ to quantify them.

Trait	Trait Image	Trait Measurement	Measurement Description
Antenna			The final five antennal segments were measured from the top to base.
Elytron			The length from the center of the causal portion to the center of the rostral portion.
Leg			The length from the pronounced top on the rostral side to the point where the tibia joins the femur.
Wing			The length from the tip of the wing to the furthest curved point on the other side.
Testes			The area of the shape of best fit around the bullbous portions.
Ovarioles			The area of the shape of best fit of the final bulbous portion.

Behavior Analysis

All behavioral analyses were quantified using BORIS, a video analysis software. The recorded mating videos were uploaded to the software and behavior was quantified following the protocol outlined in Figures 1 and 2.

Males Behavioral Analysis

Experimental males, the ones that were injected, were analyzed for their latency to antenate, latency to copulate, antennation duration, copulation duration, as well as the rate of antennation. The latency to antenate is measured as the time it took the male to tap with one of the two antennas on the elytra or head of the female. Subsequently, the latency to copulate was measured as the time it took the male to start copulation. The duration of those two behaviors is the time they spend doing them, from start to finish. Lastly, average antennation rate is a proxy measure of the rate of antennation. Average antennation was calculated by averaging the rates of 3 random periods (beginning, middle, and end) of the antennation duration. For each period of time, the number of taps of the antennae on the female's back was counted, summed, and then divided by the duration of that segment (1-3 seconds). We repeated those measures twice, one focusing on the left antennae and the other on the right antennae; finally reporting the antennation rate as the average of both average antennation rates obtained. All male behavioral analyses were conducted by two researchers and the average of their records was reported.

Female Behavioral Analysis

Experimental females, the ones that were injected, were analyzed for their latency to kick, latency to copulate, kicking duration, copulation duration, as well as their initial clutch size. Latency

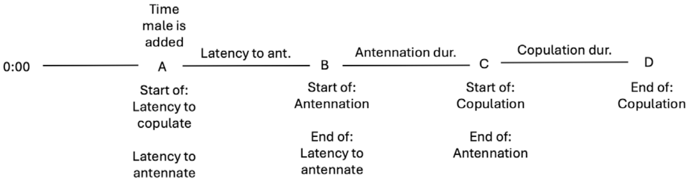

Figure 1. Males Behavioral Assay Timeline

A detailed explanation of the behavioral assay and schema followed to

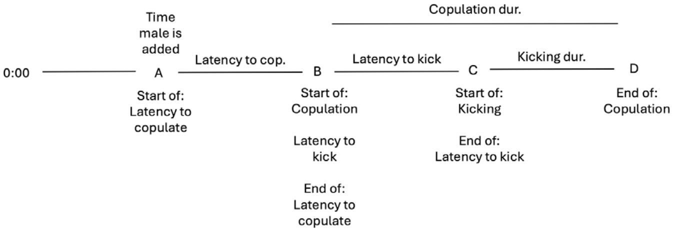

Figure 2. Females Behavioral Assay Timeline

A detailed explanation of the behavioral assay and schema followed to analyze female behavior on BORIS.

Statistical Analysis

One-way ANOVA followed by Tukey-HSD post hoc tests were used to understand the data we collected as well as any significant differences between the various treatments for both males and females. Prior to running the statistical analysis, all data were logarithmically scaled using the natural log transformation. This transformation was done to ensure that the data falls within the distribution assumptions for the parametric tests. Subsequently, statistical analysis for the morphological traits was run for the relative logarithmically transformed data. Relative data was calculated by dividing the log-transformed morphological data by the log-transformed mass for each individual.

In those one-way ANOVA tests, we explored behavioral measures such as male latency to antenate and copulate, the duration of antennation and copulation, and their antennation rate, expressed as the number of taps per second. For females, the one-way ANOVA tests explored latency to kick and copulate, the duration of those behaviors, and their initial clutch size. Lastly, we investigated morphological changes by performing six distinct morphological tests for females and males. The tests examined the size of antennae, elytra, legs, wings, gonads, and body mass.

This comprehensive approach allowed us to capture the following detailed and comprehensive understanding of how juvenile hormones influence both behavioral patterns and the physical and morphological characteristics of adult bean beetles.

Image Dataset

Through utilizing the laboratory images archive from the Barbosa Lab, I collected 1190 images across the 6 measurement classes. I followed that collection with data augmentation to populate new images from the existing ones to enhance the process of training and testing the sequential model and the automated measurement algorithm, see the data augmentation section below for reference. The number of images in each class varied after data augmentation, with the following totals: wings had 625 images, elytra had 1415 images, antennas had 1387, ovarioles had 440 images, testis had 736 images, and legs had 1375 images.

The images were all TIF type and were generated using Lecia stereoscope, digital camera, and imagining software application, as explained above, over multiple years at the Barbosa Lab in Lake Forest College.

Data Augmentation

In this research, I chose to use three different methods to augment the dataset. To maximize the original dataset, I opted to apply the 4 augmentation methods to every picture. In other words, one image would result in the creation of 4 new images in addition to itself.

The data augmentation methods that I used were rotation, horizontal flip, vertical flip, and noise addition. Firstly, the image was rotated 90 degrees clockwise using OpenCV rotate method. Later, the image was flipped both vertically then horizontally using OpenCV flip method. Lastly, the 4th new image was generated through the addition of noise by adding a random Gaussian noise matrix to each channel of the image separately.

Image preprocessing

I preprocessed the images in the dataset before training the model to optimize and standardize the process. For this purpose, I utilized the ImageDataGenerator class from the Keras library. The training data generator was configured to apply sample-wise centering and normalization, horizontal and vertical flipping, and rescaling pixel values to the range [0, 1]. For the validation and test data generators, I applied the same preprocessing steps, except for flipping, to ensure consistency in data transformation across different sets. The data generators were then used to create batches of images and their corresponding labels, which were fed into the model during the training and evaluation phases.

Programming Language and Platform

I performed all statistical analysis using R studio. Additionally, I used Python and Jupyter Notebook to conduct all image-related data manipulation, preprocessing, and image-related data manipulation, preprocessing, and model creation.

Train-Test dataset

For the LinearSVM model, I randomly used 20 images from each class to train a classifier, and the rest of the images were used for testing the classifier's accuracy. Multiple classifiers were used depending on the feature morphology, which enhanced the classification's accuracy. For instance, the classifier for wing and ovarioles extracted Local Binary Patterns (LBP) features. On the other hand, Histogram of Oriented Gradients (HOG) features were extracted from testis images and used by another classifier for testis. Lastly, contour-based features were extracted for classifying the legs, elytra, and antennas.

For the convolutional neural network linear regression model, I trained a sequential model to predict the lengths of wings using 496 training images and then tested it on 124 testing images. The CNN architecture consisted of three convolutional layers, each followed by max pooling and dropout for regularization. The model was compiled with the Adam optimizer and mean squared error loss function. The training process was conducted over 200 epochs. The CNN had two inputs: wing images and an CSV file with wing lengths and filenames.

RESULTS

Females Behavior

A one-way ANOVA test was performed on the pre-copulatory behavior of latency to copulate. The tests showed a significant change in the latency to copulate with the manipulation of JH (*F* = 7.666, *p* = 0.000935, Fig. 3a).

Similarly, four one-way ANOVA tests were used to examine the impact of JH on latency to kick, kicking duration, copulation duration, and clutch size, representing female copulatory behavior. While kicking duration did not show a significant difference across the treatment groups (*F* = 1.817, *p* > 0.05, Fig. 3d), there was a significant effect of JH treatment on the latency to kick (F = 7.414, *p* = 0.00113, Fig. 3c), copulation duration (*F* = 21.07, *p* = 5.05e-08, Fig. 3b), and the clutch size (*F* = 3.353, *p* = 0.0425, Fig. 4)

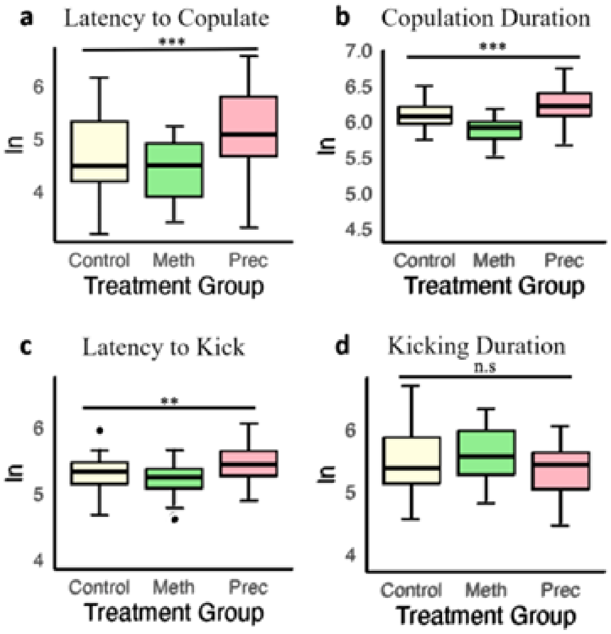

Figure 3: Behavioral Measures of Female Bean Beetles

The figure presents four boxplots illustrating key behavioral measures in female bean beetles: (a) latency to copulate, (b) copulation duration, (c) latency to kick, and (d) kicking duration. Each boxplot represents the distribution of data across treatment groups. Asterisks denote statistical significance levels: * indicates $p < 0.05$, ** indicates $p < 0.025$, and *** indicates $p < 0.001$. The results highlight significant differences in the behavioral responses of female bean beetles across experimental conditions

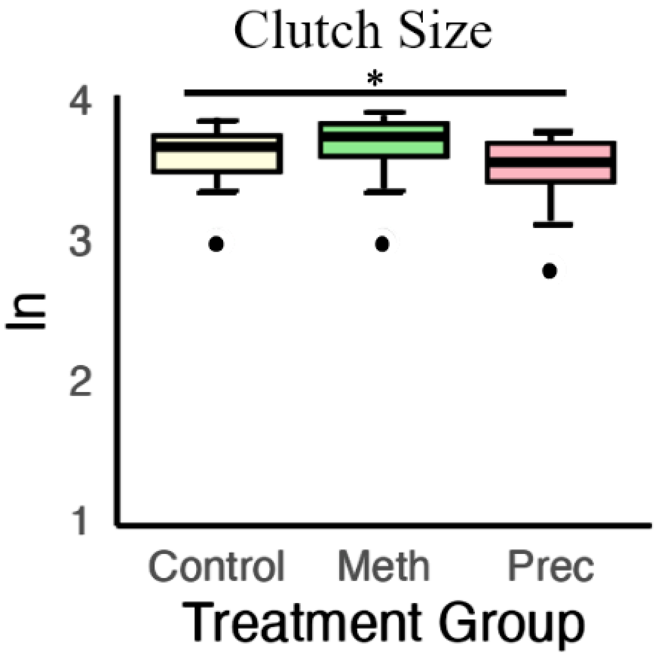

Figure 4: Clutch Size of Female Bean Beetles.

The figure presents a key behavioral measure in female bean beetles: initial clutch size across the three treatment groups. Asterisks denote statistical significance levels: * indicates $p < 0.05$.

Females Morphology

The six one-way ANOVA tests were performed on morphological traits: antennae, elytra, legs, wings, testes, and body mass. All six tests reported no difference across the treatment groups for all morphological traits, including antenna size ($F = 0.068$, $p > 0.05$, Fig. 5e), elytron size ($F = 1.399$, $p > 0.05$, Fig. 5d), leg size ($F = 0.322$, $p > 0.05$, Fig. 5c), wing size ($F = 1.318$, $p > 0.05$, Fig. 5b), ovarioles size ($F = 0.563$, $p > 0.05$, Fig. 5a), and body mass ($F = 0.403$, $p > 0.05$, Fig. 5f). These results indicate the lack of effect JH has on morphology during adulthood in female bean beetles.

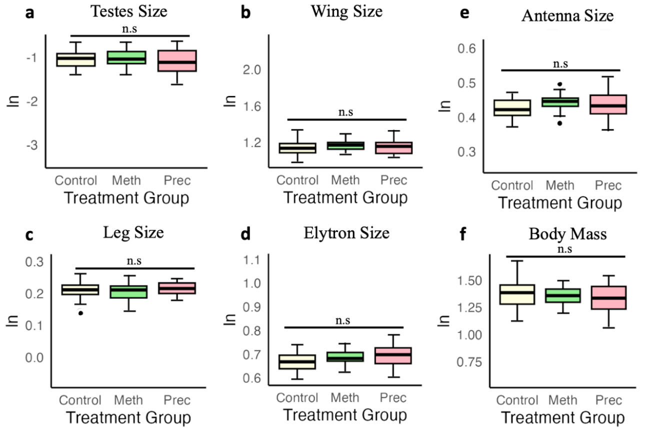

Figure 5: Morphological Measures of Female Bean Beetles

The figure displays six boxplots illustrating morphological measures in female bean beetles: (a) ovarioles size, (b) wing size, (c) leg size, (d) elytron size, (e) antennae size, and (f) body mass. Each boxplot represents the distribution of data across treatment groups. Asterisks indicate levels of statistical significance: * denotes $p < 0.05$, ** denotes $p < 0.025$, and *** denotes $p < 0.001$. The figure illustrates that juvenile hormone (JH) manipulation during adulthood does not induce significant alterations in the morphology of female bean beetles, as evidenced by the non-significant differences across the measured parameters.

Males Behavior

The four one-way ANOVA tests were performed on pre-copulatory behaviors, latency to antenate, latency to copulate, antennation duration, and antennation rate. While antennation rate did not show a significant difference across the treatment groups ($F = 0.619$, $p > 0.05$, Fig. 7), there was a significant effect of JH treatment on the latency to antennate ($F = 5.263$, $p = 0.00726$, Fig. 6c), latency to copulate ($F = 10.04$, $p = 0.000137$, Fig. 6a), and the antennation duration ($F = 3.303$, $p = 0.0422$, Fig. 6d)

Similarly, a one-way ANOVA test was used to examine the impact of JH on copulation duration, representing male copulatory behavior. The test showed no change in the duration of the copulatory behavior with the manipulation of JH ($F = 0.403$, $p > 0.05$, Fig. 6b).

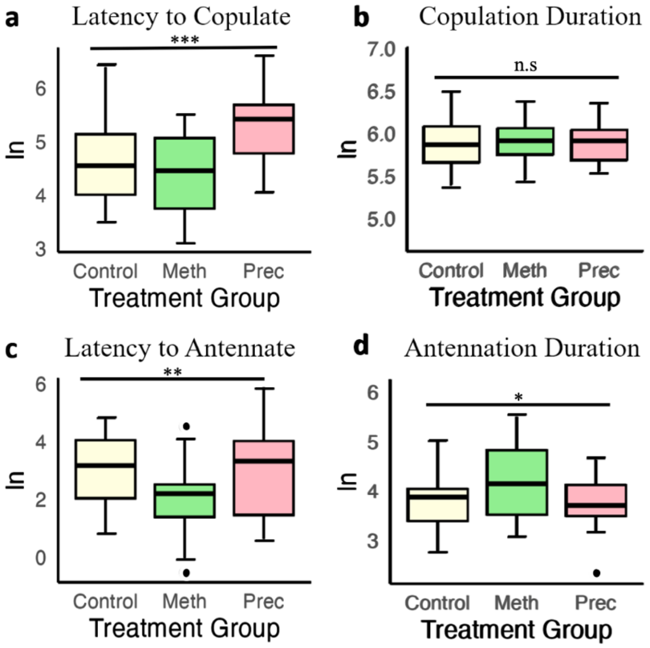

Figure 6: Behavioral Measures of Male Bean Beetles

The figure presents four boxplots illustrating key behavioral measures in female bean beetles: (a) latency to copulate, (b) copulation duration, (c) latency to antenate, and (d) antennation duration. Each boxplot represents the distribution of data across treatment groups. Asterisks denote statistical significance levels: * indicates $p < 0.05$, ** indicates $p < 0.025$, and *** indicates $p < 0.001$. The results highlight significant differences in the behavioral responses of male bean beetles across experimental conditions

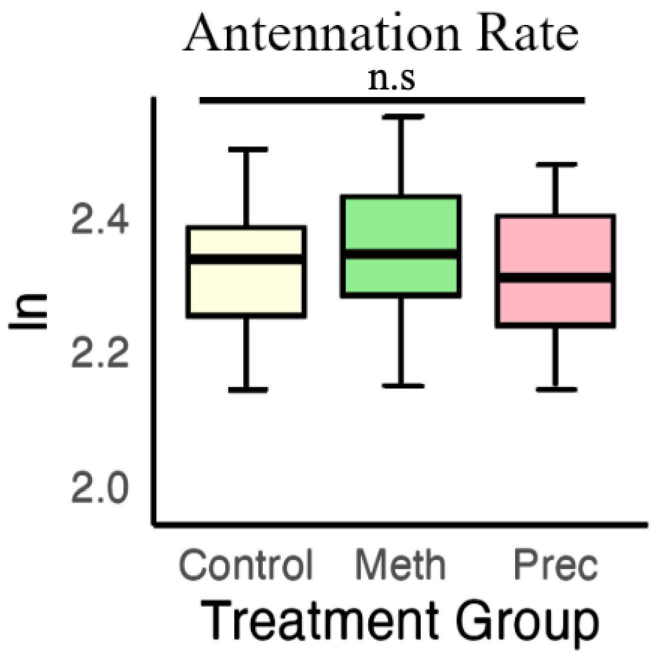

Figure 7: Antennation Rate of Male Bean Beetles

The figure presents a key behavioral measure in male bean beetles: average antennation rate across the three treatment groups. n.s denote statistical non-significance.

Males Morphology

The six one-way ANOVA tests were performed on morphological traits: antennae, elytra, legs, wings, testes, and body mass. All six tests reported no difference across the treatment groups for all morphological traits, including antenna size (*F* = 0.185, *p* > 0.05, Fig. 8e), elytron size (F = 0.484, p > 0.05, Fig. 8d), leg size (*F* = 0.126, *p* > 0.05, Fig. 8c), wing size (*F* = 0.273, *p* > 0.05, Fig. Yb), testes size (*F* = 0.184, *p* > 0.05, Fig. 8a), and body mass (*F* = 0.330, *p* > 0.05, Fig. 8f). These results indicate the lack of effect JH has on morphology during adulthood in male bean beetles.

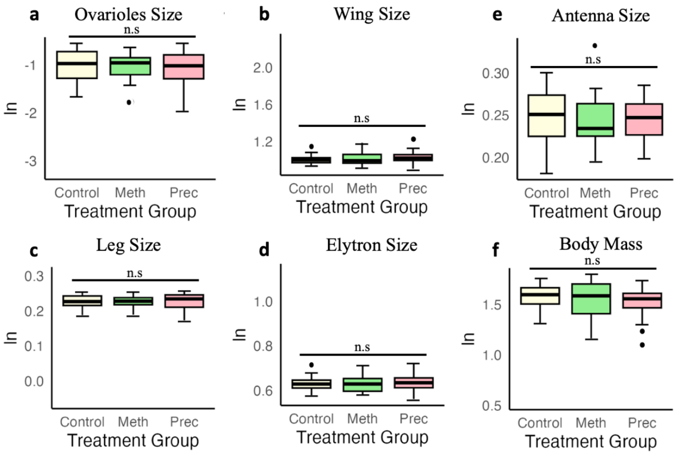

Figure 8: Morphological Measures of Male Bean Beetles

The figure displays six boxplots illustrating morphological measures in male bean beetles: (a) testes size, (b) wing size, (c) leg size, (d) elytron size, (e) antennae size, and (f) body mass. Each boxplot represents the distribution of data across treatment groups. Asterisks indicate levels

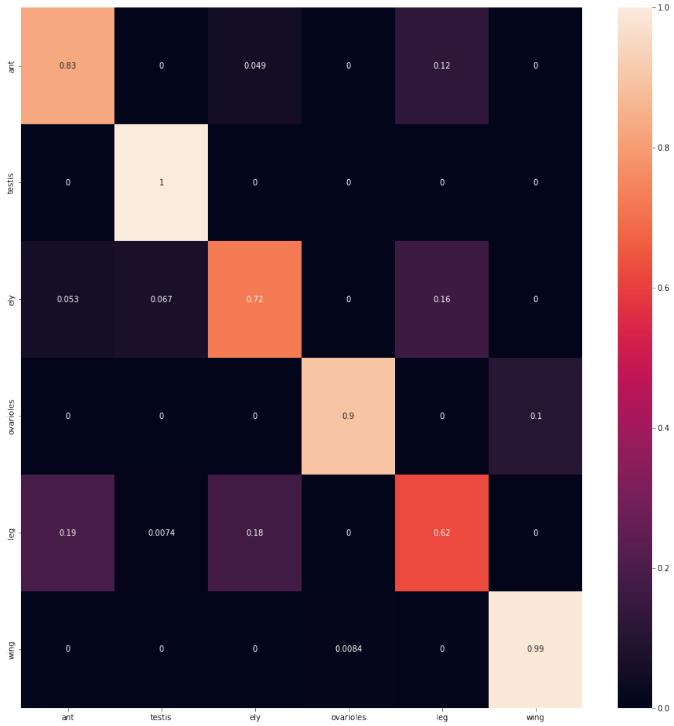

Figure 9: Confusion Matrix of 6 Classifiers Performance

The confusion matrix provides a comprehensive visualization of the performance of six classifiers across multiple classes. Each row of the matrix represents the actual classes, while each column represents the predicted classes. The elements of the matrix indicate the percentage of instances belonging to each class that were correctly or incorrectly classified by the respective classifiers. A lighter-colored box indicates a higher classification accuracy.

Automated Morphological Measurement

The performance of the CNN model was evaluated on both the training and test datasets. The following metrics were obtained: Mean Squared Error (MSE) on the test set: 0.01897, Root Mean Squared Error (RMSE) on the test set: 0.13772, Mean Absolute Error (MAE) on the test set: 0.11006. The model demonstrated a reasonable degree of accuracy in predicting the lengths of beetle wings, with an R-squared value of 0.63475 indicating that approximately 63.48% of the variance in the actual lengths is explained by the model's predictions

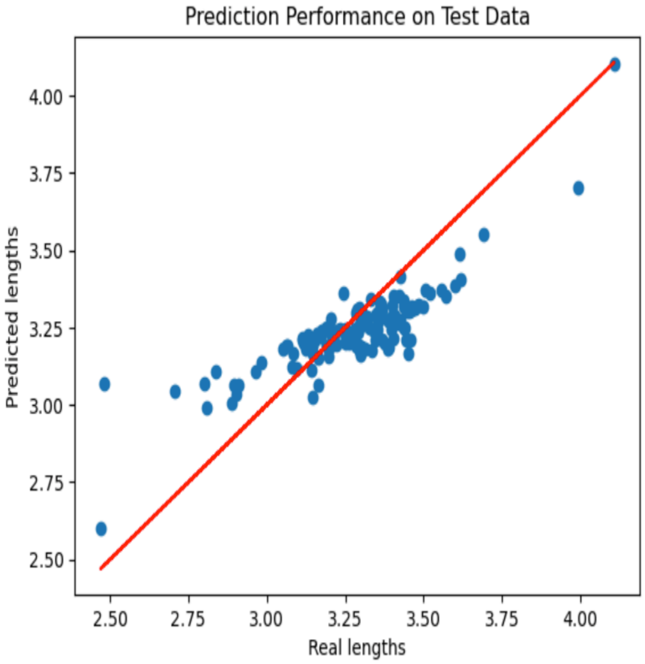

Figure 10. Scatter Plot of Actual vs Predicted Lengths of Test Data

This scatter plot illustrates the performance of the convolutional neural network (CNN) model on the test data. Each point represents an individual prediction, with the actual lengths on the x-axis and the predicted lengths on the y-axis. The red line indicates the line of perfect prediction where the predicted lengths would exactly match the actual lengths. The clustering of points around this line suggests a positive correlation, indicating the model's effective predictive ability.

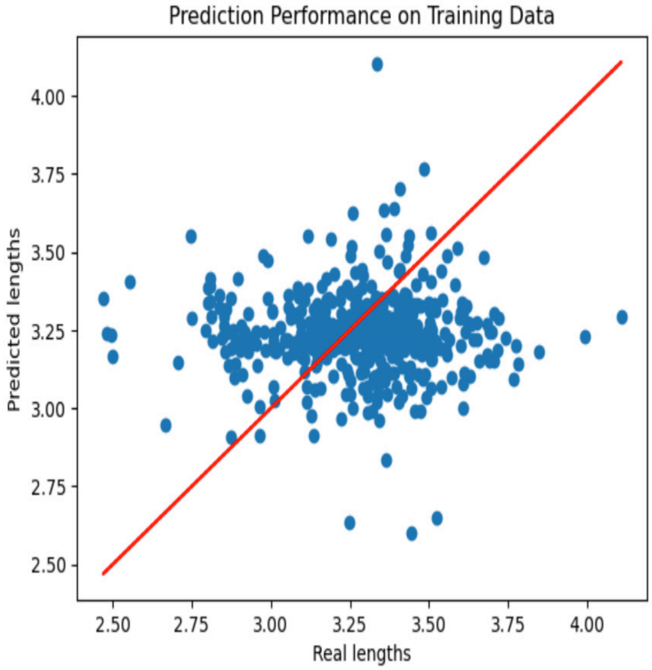

Figure 11. Scatter Plot of Actual vs Predicted Lengths of Training Data

This scatter plot displays the CNN model's prediction accuracy on the training data. Each blue point corresponds to a single observation, with the x-axis showing the true lengths and the y-axis depicting the model's predictions. The red line represents the ideal scenario where predictions would be precisely equal to the true values. The distribution of points around the line demonstrates the model's learning trend during the training phase.

DISCUSSION

Section 1: Hormonal Manipulation

1a) Findings, Impact, and Importance

This research illustrated that an increase in Juvenile Hormone titer in both female and male bean beetles during early adulthood increases the intensity of their copulation behaviors without affecting their morphological traits. These findings are consistent with the expectations of previous research done in the Barbosa lab that investigated the role of JH in larvae. Specifically, previous research showed that the manipulation of JH in the larvae stage leads to morphological manipulation with little to no behavioral alterations. Thus, we expected the manipulation of JH during the adult stage to lead to the opposite. These expectations were met in the results of this research. More specifically, this research found that females' copulation duration and the time it takes them to start copulating (latency to copulate) and kicking (latency to kick) decreased with higher JH levels (Methoprene). Yet, the number of eggs they laid in a week (initial clutch size) increased in comparison to individuals treated with the JH inhibitor, Precocene. These findings indicate a potential trade-off between copulation behaviors and reproductive output. In other words, females that spent less time copulating had better success with laying more eggs. In males, a similar pattern was detected. Individuals treated with the JH analog started copulating and antennating more quickly than JH inhibitor-treated individuals. This suggests the distinct roles of different JH-related compounds in regulating the initiation of reproductive behaviors in males and females. Furthermore, while their antennation rate remained unchanged, their antennation duration increased. This potentially suggests some refined communication or mate recognition strategies in response to hormonal changes in bean beetles.

These findings illustrate a novel understanding of the role of JH in bean beetles' development and its influence in shaping their sexual and reproductive behaviors. Moreover, they outline a distinct separation between behavioral changes and morphological traits. This separation highlights the complexity of the hormonal-behavioral relationship and the need for a comprehensive understanding of the connections between hormones, behavior, morphology, and evolutionary processes in the animal kingdom. It also underscores the importance of timing in development, as it relates to the distinct roles of hormonal organizational and activational effects in development. Organizational effects, which occur during early critical developmental periods, lay the foundation for future physiological and behavioral patterns, while activational effects, which occur later in life, trigger the activation of specific behaviors or physiological responses. Therefore, organizational effects create permanent changes in the neural substrates that control behaviors, which lead to the development of the nervous system and behavior along separate developmental pathways (Elekonich & Robinson, 2000). These organizational effects ultimately influence the manifestation of behaviors later in life by causing variation in the development of individuals within particular pathways. On the other hand, activational effects modify the neural activities within the pre-existing pathways during adulthood. They influence behaviors in response to environmental or social conditions. However, they are typically reversible (Elekonich & Robinson, 2000). It is crucial to understand the influence of those effects on trade-offs and behavior. Thus, this research reinforces the need for a deeper understanding of the mechanisms underlining the relationship between trade-offs and hormones, particularly in the context of their organizational and activational effects throughout the developmental stages.

1b) Limitations

While the study provides some insightful results, there are some limitations that could have influenced the findings and should be considered. Firstly, attempting to quantify both behavior and morphology to assess the role of JH forced us to operationalize those definitions. Those previously and consistently used definitions could influence the interpretation of the findings. For example, we measure the size of the wing, elytra, and leg by measuring their length instead of their area to ensure both consistency and less confounding variables. However, measuring their area could lead to better insight into the morphological alterations. In particular, area measurements, unlike length measurements, provide a 2-dimensional representation of the body part as it records the width and length. This could provide better insight into understanding the hormonal effects on the overall size, both width and length. Secondly, JH manipulations were achieved through manual injections using a Hamilton syringe. Those injections may have introduced a stressor, that was mitigated by a 24-hour rest period. Alternatively, there may have been some variations in the quantity of solution that was absorbed by the individual beetles, leading to some inconsistency in the experimental procedure. Lastly, we used the same dosage for all individuals without correcting for body size. This could have led to variations in the hormonal effects observed because smaller individuals may have experienced a higher dose per milligram of body mass compared to larger individuals and vice versa.

1c) Future Studies

The findings of this study pave the way for a variety of future directions in exploration of the role of JH in bean beetles. For instance, to further understand mating behaviors, future studies could focus on reintroducing JH-analog in males and females after initially inhibiting its production. This experiment would highlight the possibility for the reversibility and plasticity of JH influence on copulation behavior and mating output. One potential issue with such a study would be introducing a second mating session, which is difficult to occur for bean beetles. Additionally, investigating the temporal aspects of JH effects is crucial for a comprehensive understanding. In this study, JH manipulation occurred within the first 12 hours after emergence. Future studies could explore how the timing of JH manipulation during different post-emergence stages influences mating behaviors and reproductive outcomes. This can highlight any developmental windows for behavioral changes that bean beetles have. Furthermore, future studies focusing on exploring the long-term effects of Juvenile Hormone (JH) manipulation across multiple generations could offer us valuable insights into the generational impact and evolutionary trajectory of bean beetles.

More importantly, the findings of this study highlight the importance of further exploring the complex relationship between hormones and behavior. In an attempt to do so, future research can focus on exploring the interactions between JH and other hormones that contribute to the network of hormonal influences on sexual maturation and mating behaviors. These studies can investigate the interactions of ecdysteroids, insulin-like peptides (ILPs), and neuropeptide in addition to JH. Ecdysteroids is crucial for metamorphosis; whereas ILPs influence growth, development, and reproduction; and neuropeptides are key for physiological processes in insects including the regulation of JH. Therefore, expanding our knowledge of the network of insects' endocrine system is essential to grasp a holistic understanding of bean beetles' sexual maturation and behavior. Besides, examining the presence of any trade-offs or evolutionary contexts within that network allows for a better appreciation of the adaptive processes within these species.

Section II: Automated Measurement ML Model

2a) Findings, Impact, and Importance

It has also generated a high level of accuracy in classifying 6 complex bean beetle morphological features using 6 LinearSVC models. Those models were unique based on the morphological features in hand. For instance, the testis model extracted HOG feature descriptors, whereas 2 models used LBP feature descriptors to classify wings and ovarioles. Lastly, 3 models used the concatenated feature descriptors of contour-based features and color histogram to classify legs, elytra, and antennas. The models showed the following accuracies 100%, 99%, 90%, 62%, 72%, and 83% for classifying the testis, wings, ovarioles, legs, elytra, and antennas, respectively. Therefore, the classifiers have an overall accuracy of 84%. This indicates a high level of ability for the computer to classify morphological features' images, especially if they are images of gonads or wings. Nonetheless, the classifiers are finding it difficult to classify images with tail-like shapes in them, as in the antenna and the legs. Similarly, images of the wing covers (elytra) have thread-like shapes in them due to the mounting technique in the laboratory. Therefore, I predict that with better mounting, elytra's classification would become more accurate.

These findings shown in the results section illustrate the best outcomes from multiple models developed as well as a variation of descriptors and parameters used. It is vital to understand the difference between those models, what worked best in each one of them, as well as the impact each one had on my research. The journey to achieving an overall accuracy of 84% started off with a model with an accuracy of 40% at classifying the classes correctly. This model, labeled Model1, used Local Binary Patterns (LBP) to extract features from the images. A closer look at the performance across the different classes revealed significant variations: while Model1's LBP excelled in identifying wings and ovarioles with accuracies of 89% and 99%, respectively, it failed drastically with other classes such as antenna (5.9%), testis (8.4%), elytra (5.7%), and legs (0.3%).

This uneven performance prompted me to incorporate Histogram of Oriented Gradients (HOG) in Model2. HOG is known for capturing edge and gradient structures that could complement the texture information from LBP. This addition improved the classification results by 22%. The overall model accuracy jumped to 62%, with notable improvements in the previously underperforming class of testis whose accuracy rose to 81%. However, despite these gains, some classes like antenna (43%), elytra (30%), and legs (26%) remained poorly classified, prompting further exploration and improvements in the model. Therefore, in Model3, I introduced contour-based features for the antenna, elytra, and legs, while retaining HOG for the testis and LBP for wings and ovarioles. This adjustment led to a further increase in accuracy to 69%. The new contour features significantly improved the antenna classification to 87%, but elytra and legs continued to present challenges, with leg classification accuracy at 41% and elytra at 0%. Notably, there was a high rate of misclassification with elytra being confused as antenna 59% of the time and legs as antenna 75% of the time.

To address these persistent issues, I experimented with color histograms in Model4, although this change reduced the overall accuracy slightly to 67%. The model still struggled with distinguishing between similar-looking classes, leading to a continued poor performance in classifying elytra.

Finally, in Model5, I adopted a concatenated approach combining the strengths of different descriptors for the problematic classes. This strategy significantly boosted the model's effectiveness, resulting in an overall accuracy of 84%. This model achieved impressive class-specific accuracies: antenna (83%), testis (100%), elytra (72%), ovarioles (90%), legs (62%), and wings (99%).

This iterative process of refining the feature descriptors not only enhanced the model's accuracy but also highlighted the importance of choosing appropriate descriptors based on the specific characteristics of each class. For instance, I predict that LBP's effectiveness at capturing fine texture details is likely what contributed to its high accuracy in classifying wings and ovarioles. These classes have distinct textural patterns that LBP could efficiently detect and differentiate.

Similarly, HOG descriptors are particularly excellent at detecting edge directions and gradients, which proved beneficial for classes such as the testis where structural edges and shape contours are more pronounced and specific to their shape. This helped in distinguishing the testis from other classes and allowed them to stand out. However, HOG was less effective for classes like elytra and legs, where the edges and gradients might be more overwhelmed by the background variations as well as the similarities across each other. Additionally, contour-based features are specifically designed to capture boundary shapes and contours while dicarding information about the color of the images. This unique approach significantly improved the classification of the antenna, a class where the shape and silhouette are distinctive when highlighting its detials. Nonetheless, this advantageous characteristic failed to effectively classify the elytra and legs. This might be due to the similarity in contours or overlapping features among these classes, which led to high misclassification rates. Contrarily, color histograms can provide useful classification signals based on the unique color distributions of the images. The poor performance in classifying elytra suggests that color histograms alone are insufficient to capture the necessary distinguishing features. Therefore, the concatenated features allowed for the combination of multiple descriptors, which leverages the strengths of each to address their individual weaknesses. For instance, the use of color histogram and contour-based features allowed the model to better classify and effectively identify legs, elytra, and antenna.

The CNN model's success in this study is a critical milestone in the application of deep learning to biological research, offering significant advancements in the automated measurement of morphological features. The model's capabilities were particularly exemplified by its precision in assessing straight-line measurements on beetle wings from a diverse image dataset. Notably, the model accomplished this with a limited number of images by learning from augmented data, illustrating its efficacy in feature recognition and length estimation under constrained conditions. These findings have considerable implications for the field, as they indicate that even with limited data, neural network models can be trained to perform complex tasks with a high degree of accuracy. These computational capabilities not only conserve valuable research time but also reduce the potential for human error in morphometric analyses, leading to more reliable and reproducible studies. The importance of such a development cannot be overstated, particularly in fields where precision is vital. This is why it is crucial to invest resources and time to better equip such a model.

The development of my CNN regression model was characterized by an iterative process of tuning and refinement, aiming to enhance the accuracy of predicting beetle wing lengths. The journey began with a basic sequential neural network and involved systematic modifications to various model parameters, such as batch sizes, epochs, kernels, and the architecture itself, including the addition or removal of layers and adjustments to pooling layer types.

The CNN regression model was adjusted and built similarly to the classes' classifier, where each subsequent model version aimed to address specific shortcomings identified in the preceding versions. In particular, I focused on the gradual decrease in MSE, RMSE, and MAE across the models, which indicates effective enhancements in model precision and accuracy. I also focused on increasing the R-squared value to demonstrate an improved ability of the models to explain a greater proportion of variance in the wing measurements.

The first model built, Model1, set a baseline with a Mean Squared Error (MSE) of 0.075 and an R-squared value of 0.30. These results yielded poor length predictions that fell far from the actual lengths of wings. Therefore, in Model2, I increased the epochs to test for lack of convergence due to limitations in the number of iterations and learning steps. Model2 performed better than Model1, with an MSE of 0.052 and an R-squared of 0.40. However, this improvement was not enough to reach accurate and reliable predictions. Therefore, Model3 incorporated the addition of more complexity by adding more convolutional layers and dropout layers. However, despite these changes, Model3 resulted in an unexpected decrease in performance. Particularly, there was a drop in the MSE to 0.60 and the R-squared to 0.32. This highlighted some of the challenges of managing model complexity, learning effectiveness, and sensitivity to changes.

Model4 tried to address this setback by retracting the changes on the convolutional and dense layers and instead implementing alterations on the pooling layers types from max to average and further increased the dropout rates. By inducing those changes, I aimed to refine feature extraction and enhance the model's learning. Unfortunately, these changes also led to a degradation in performance, showcasing another example of the difficulty in fine-tuning CNN architectures for specific measurement tasks. Nonetheless, Model5 represented the culmination of the various refinements and was designed to integrate the most effective features and strategies from the previous models while incorporating new optimizations to address prior shortcomings.

Model5, which yielded the best-reported results, begins with a convolutional layer that has 32 filters with the capability to process input images of size 480x640. This layer is critical for initial feature extraction, such as edges and simple textures. Following the first layer, a more complex convolutional layer with 128 filters was added, which allowed for the extraction of higher-level features from the reduced spatial dimensions of the previous pooling layer. Additionally, a third convolutional layer with 64 filters further refines the feature map to capture even more detailed attributes crucial for accurate morphological assessments. As mentioned, in between each convolutional layer is followed by a max pooling layer and a dropout layer. The max pooling layers reduce the spatial dimensions of the feature maps, which makes feature detection computationally less costly and helps in making the detection of features invariant to scale and orientation. Similarly, the addition of dropout layers after each pooling step and dense layers randomly disables a fraction of the neurons during training, preventing overfitting and ensuring that the model generalizes well to new, unseen data. Lastly, the model had flattening and dense layers that aim to reduce the number of neurons to combine the features into patterns that are more abstract and representational of the input data. For instance, the flattening layer converts the 2D feature maps into a 1D vector, making it possible to feed into dense layers. The subsequent dense layers progressively reduce the number of neurons, first to 64 and then to 16, allowing for the combination to occur. Finally, the final dense layer reduces the output to a single neuron that predicts the length of beetle wings, which represents the regression output.

2b) Limitations

The tool created in this research is inherently limited due to biases and limitations within the dataset itself. Therefore, the models can get as good as their dataset. The dataset I used is for a bean beetle population that was used in a research laboratory. Therefore, I had to populate images out of the pre-existing dataset due to the limited number of images available. This process could have been eliminated by having access to a big dataset of images, which would have reduced the biases in the image set. Due to the need for various feature descriptors to train the classifiers, my models are time-consuming. Therefore, they require a relatively high level of computational power to train.

On the other hand, the wing measurement convolutional neural network model also has some limitations. It was designed to measure straight lines, which assumes that the wings are fully spread and properly aligned in the image. In cases where the beetle's wings are not fully extended or overlapped, the model might not accurately measure the length. Furthermore, the model's accuracy depends on the precision of the initial annotations used for training. If the annotated lengths were not exact due to human error, this could have introduced an additional source of error that the model would propagate. Finally, due to computational constraints, the model was trained for a fixed number of epochs which may not have been sufficient to reach the optimal performance. With more time and resources, further tuning of the hyperparameters and extended training could potentially improve the model's performance.

2c) Future Studies

This work highlights the possibility of further utilizing machine learning in research. It also further underscores the importance of utilizing intersectional knowledge and skills to optimize time and energy. It also paves the way for future enhancements and other applications. For instance, future studies could utilize intersectional collaboration to develop better behavior assay analysis tools. This would better standardize the process and lead to a decrease in human inconsistencies. Similarly, building a better classification tool would allow the researchers to rely on the computer to sort through their images and organize their folders. This means that future work could focus on building better feature descriptors for the legs, elytra, and antenna where the classifiers focus on more prominent parts of those morphological traits.

While my methodology has demonstrated promising results, there is always room for refinement and enhancement. Future studies could focus on optimizing various aspects of the pipeline, including alterations in the parameters, data preprocessing techniques, and classifier architectures. They should also focus on refining the process of training, where the manually measured images could be measured more precisely to reduce learning noise. Besides, future studies should focus on covering the measurement process for all of the morphological traits of interest in the Barbosa Lab. Afterward, I'd be interested in applying my model and classifier to other species and beetle populations to examine the accuracy of their results. Ideally, these models would be able to adapt and generalize their power to a bigger dataset than the one in hand.

Conclusion

This research has provided insights into the role of Juvenile Hormone (JH) in the sexual and reproductive behaviors of adult bean beetles. It has also introduced the development of automated classification and a wing measurement tool using machine learning and neural network techniques. The findings of this research highlight the fascinating relationship between hormones, behavior, and trade-offs with an emphasis on the importance of timing within this relationship. In particular, this research showed the impact of juvenile hormones during adulthood, where a higher level of JH induced an intensified behavioral activity in both male and female bean beetles without significant impact on their morphology. Thus, the findings suggest that JH plays a critical role in regulating sexual behaviors in both male and female bean beetles, with significant effects observed in latency to copulate, latency to kick, copulation duration, and clutch size for females, and latency to antennate, latency to copulate, and antennation duration for males.

The development of the automated measurement tool demonstrates the potential of machine learning in streamlining many processes and analysis stages in research. Further, this research has shown the possibility of using classification for categorizing and identifying various traits and areas of focus in research, which can optimize time. The classifiers demonstrate high accuracy in classifying testes, wings, and ovarioles, with lower accuracy observed for legs, elytra, and antennas. The development of those LinearSVC models for classifying morphological features and a sequential neural network model for automating wing length measurement represents a significant step forward in the automation of morphological measurements, with potential applications in various fields of biological research. However, this research highlights the importance of consistency of data collection in laboratories as limitations to those models arise with limited datasets, making them limited in their precision. Therefore, an expansive dataset would generate more generalizable, complex, and applicable models.

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APPENDIX

1) R code for statistical analysis

1a) Males one-way ANOVA test and post hoc test

~~~~
```{r}

selected_vars <- c(“LOG_M_MASS”, “LOG_AVG_ANT_RATE”,”LOG_LAT_ANT”,”LOG_DUR_ANT”,”LOG_LAT_COP”,”LOG_DUR_COP”)

# a list to store ANOVA results

anova_results <- list()

tukey_results <- list()

plots <- list()

# one-way ANOVA for each variable

for(var in selected_vars) {

  formula <- as.formula(paste(var, “~”, “Treatment”))

  anova_result <- aov(formula, data = JH23_males)

  tukey_result <- TukeyHSD(anova_result)

  tukey_results[[var]] <- tukey_result

  anova_results[[var]] <- summary(anova_result)

  plot <- ggplot(JH23_males, aes(x = Treatment, y = !!sym(var))) +

    geom_boxplot(fill = “lightblue”) +

    labs(x = “Treatment Group”, y = var, title = var)

  plots[[var]] <- plot

}

# summaries of the ANOVA results for each variable

for (var in selected_vars) {

  cat(“one-way ANOVA for”, var, “\n”)

 print(anova_results[[var]])

  cat(“\n”)

  cat(“Post Hoc Test for”, var, “\n”)

  print(tukey_results[[var]])

  cat(“\n”)

}
~~~~

~~~~
```{r}

selected_vars <- c(“LOG_M_MASS”, “relative_LOG_Testes”,”relative_LOG_Antenna”,”relative_LOG_Wing”,”relative_LOG_Leg”,”relative_LOG_Elytra”)

# a list to store ANOVA results

anova_results <- list()
~~~~

~~~
tukey_results <- list()


# one-way ANOVA for each variable
for(var in selected_vars) {
  formula <- as.formula(paste(var, “~”, “Treatment”))
  anova_result <- aov(formula, data = JH23_males)
  tukey_result <- TukeyHSD(anova_result)
  tukey_results[[var]] <- tukey_result
  anova_results[[var]] <- summary(anova_result)
}
# summaries of the ANOVA results for each variable
for (var in selected_vars) {
  cat(“one-way ANOVA for”, var, “\n”)
  print(anova_results[[var]])


cat(“\n”)
  cat(“Post Hoc Test for”, var, “\n”)
  print(tukey_results[[var]])
  cat(“\n”)
}
```
~~~

1b) males box plots

~~~
```{r}
group_colors <- c(“Control” = “lightyellow”, “Meth” = “lightgreen”, “Prec” = “lightpink”)
plots <- list()
selected_vars <- c(“LOG_M_MASS”, “LOG_AVG_ANT_RATE”, “LOG_LAT_ANT”, “LOG_DUR_ANT”, “LOG_LAT_COP”, “LOG_DUR_COP”)
# Function to create, print, and save plots with a specified aspect ratio, removing outliers create_print_save_plots_no_outliers <- function(log_col, plot_width, y_range) {
  # Calculate the IQR
  Q1 <- quantile(JH23_males[[log_col]], 0.25)
  Q3 <- quantile(JH23_males[[log_col]], 0.75)
  IQR <- Q3 - Q1
  # Define the lower and upper bounds for identifying outliers
  lower_bound <- Q1 - 1.5 * IQR
  upper_bound <- Q3 + 1.5 * IQR
  # Filter the data to exclude outliers
  data_filtered <- JH23_males[JH23_males[[log_col]] >= lower_bound & JH23_males[[log_col]] <= upper_bound, ]
   plot <- ggplot(data_filtered, aes(x = Treatment, y = !!sym(log_col), fill = Treatment)) + geom_boxplot(color = “black”) +
    scale_fill_manual(values = group_colors) +
    labs(x = “Treatment Group”, y = “ln”, title = log_col) +
    theme_minimal() +
    theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(), axis.line = element_line(color = “black”)) +
    ylim(y_range) +
    guides(fill = FALSE)
  # Calculate the dimensions for saving the plots
  plot_height <- 2
  plot_width <- 2
  # Print the plot
  print(plot)
  # Save the plot with the specified dimensions
    ggsave(paste0(log_col, “_plot_males.tiff”), plot = plot, width = plot_width, height = plot_height)
}
# Iterate through selected variables and create/print/save plots without outliers
for (log_col in selected_vars) {
  y_range <- c(min(JH23_males[[log_col]]), max (JH23_males[[log_col]])) # Adjust the y-axis range as needed
  create_print_save_plots_no_outliers(log_col, plot_width, y_range)
}
```


``{r}
=group_colors <- c(“Control” = “lightyellow”, “Meth” = “lightgreen”, “Prec” = “lightpink”)
~~~

~~~
plots <- list()

# Define the selected variables selected_vars <- c(“LOG_M_MASS”,

“relative_LOG_Testes”,”relative_LOG_Antenna”,”relative_LOG_Wing”,”relative_LOG_Leg”,”relative_LOG_Elytra”)

# Function to create, print, and save plots with a specified aspect ratio, removing outliers

create_print_save_plots_no_outliers <- function(log_col, plot_width, y_range) {

 # Calculate the IQR

 Q1 <- quantile(JH23_males[[log_col]], 0.25)

 Q3 <- quantile(JH23_males[[log_col]], 0.75)

 IQR <- Q3 - Q1

 # Define the lower and upper bounds for identifying outliers

 lower_bound <- Q1 - 1.5 * IQR

 upper_bound <- Q3 + 1.5 * IQR

 # Filter the data to exclude outliers

  data_filtered <- JH23_males[JH23_males[[log_col]] >= lower_bound & JH23_males[[log_col]] <= upper_bound, ]

  plot <- ggplot(data_filtered, aes(x = Treatment, y = !!sym(log_col), fill = Treatment)) + geom_boxplot(color = “black”) +

   scale_fill_manual(values = group_colors) +

   labs(x = “Treatment Group”, y = “ln”, title = log_col) +

   theme_minimal() +

  theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(), axis.line = element_line(color = “black”)) +

   ylim(y_range) +

   guides(fill = FALSE)

 # Calculate the dimensions for saving the plots

 plot_height <- 2

 plot_width <- 2

 # Print the plot

 print(plot)

 # Save the plot with the specified dimensions

  ggsave(paste0(log_col, “_plot_males.tiff”), plot = plot, width = plot_width, height = plot_height)

}

# Iterate through selected variables and create/print/save plots without outliers for (log_col in selected_vars) {

  y_range <- c(min(JH23_males[[log_col]]), max(JH23_males[[log_col]])) # Adjust the y-axis range as needed create_print_save_plots_no_outliers(log_col, plot_width, y_range)

}

```
~~~

1c) Females one-way ANOVA test and post hoc test

~~~
```{r}

selected_vars <- c(‘LOG_clutch’, “LOG_LAT_COP”, ‘LOG_DUR_COP’, “LOG_LAT_KICK”, ‘LOG_DUR_KICK’)

# grouping_var <- “Treatment”

# a list to store ANOVA results

anova_results <- list()

tukey_results <- list()

# one-way ANOVA for each variable

for(var in selected_vars) {

 formula <- as.formula(paste(var, “~”, “Treatment”))

 anova_result <- aov(formula, data = JH23_females)

 tukey_result <- TukeyHSD(anova_result)

 tukey_results[[var]] <- tukey_result

 anova_results[[var]] <- summary(anova_result)

}

# summaries of the ANOVA results for each variable

for (var in selected_vars) {

 cat(“one-way ANOVA for”, var, “\n”)

 print(anova_results[[var]])

 cat(“\n”)

 cat(“Post Hoc Test for”, var, “\n”)

 print(tukey_results[[var]])

 cat(“\n”)

}

```

```{r}
~~~

~~~
selected_vars <- c(“LOG_F_MASS”, “relative_LOG_Ovarioles”,”relative_LOG_Antenna”,”relative_LOG_Wing”,”relative_LOG_Leg”,”relative_LOG_Elytra”)

# a list to store ANOVA results

anova_results <- list()

tukey_results <- list()

# one-way ANOVA for each variable

for(var in selected_vars) {

  formula <- as.formula(paste(var, “~”, “Treatment”))

  anova_result <- aov(formula, data = JH23_females)

  tukey_result <- TukeyHSD(anova_result)

  tukey_results[[var]] <- tukey_result

  anova_results[[var]] <- summary(anova_result)

}

# summaries of the ANOVA results for each variable

for (var in selected_vars) {

  cat(“one-way ANOVA for”, var, “\n”)

  print(anova_results[[var]])

  cat(“\n”)

  cat(“Post Hoc Test for”, var, “\n”)

  print(tukey_results[[var]])

  cat(“\n”)

}

```
~~~

1d) Females Box plots

~~~
```{r}

group_colors <- c(“Control” = “lightyellow”, “Meth” = “lightgreen”, “Prec” = “lightpink”)

plots <- list()

selected_vars <- c(‘LOG_clutch’, “LOG_LAT_COP”, ‘LOG_DUR_COP’, “LOG_LAT_KICK”, ‘LOG_DUR_KICK’)

# Function to create, print, and save plots with a specified aspect ratio, removing outliers

create_print_save_plots_no_outliers <- function(log_col, plot_width, y_range) {

  # Calculate the IQR

Q1 <- quantile(JH23_females[[log_col]], 0.25, na.rm = TRUE)

  Q3 <- quantile(JH23_females[[log_col]], 0.75, na.rm = TRUE)

  IQR <- Q3 - Q1

  lower_bound <- Q1 - 1.5 * IQR

  upper_bound <- Q3 + 1.5 * IQR

  # Filter data to exclude outliers

  data_filtered <- JH23_females[JH23_females[[log_col]] >= lower_bound & JH23_females[[log_col]] <= upper_bound, ]

  # Filter data to exclude rows with NA values in log_col or Treatment

   data_filtered <- data_filtered[complete.cases(data_filtered[, c(“Treatment”, log_col)]), ]

  plot <- ggplot(data_filtered, aes(x = Treatment, y = !!sym(log_col), fill = Treatment)) +

    geom_boxplot(color = “black”) +

    scale_fill_manual(values = group_colors) +

    labs(x = “Treatment Group”, y = “ln”, title = log_col) +

     theme_minimal() + theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(), axis.line = element_line(color = “black”)) +

    ylim(y_range) +

    guides(fill = FALSE)

  plot_height <- 2

  plot_width <- 2

  print(plot)

    ggsave(paste0(log_col, “_plot.tiff”), plot = plot, width = plot_width, height = plot_height)

}

# Iterate through selected variables and create/print/save plots without outliers

for (log_col in selected_vars) {

  y_range <- c(min(JH23_females[[log_col]], na.rm = TRUE), max(JH23_females[[log_col]], na.rm = TRUE))  # Adjust the y-axis range as needed

  create_print_save_plots_no_outliers(log_col, plot_width, y_range)

}



```
~~~

~~~~
```{r}

group_colors <- c(“Prec” = “lightpink”, “Control” = “lightyellow”, “Meth” = “lightgreen”)

plots <- list()

selected_vars <-

c(“relative_LOG_Ovarioles”,”relative_LOG_Antenna”,”relative_LOG_Wing”,”relative_LOG_Leg”,”relative_LOG_Elytra”)

# Function to create, print, and save plots with a specified aspect ratio, removing outliers create_print_save_plots_no_outliers <- function(log_col, plot_width, y_range) {

  # Calculate the IQR

  Q1 <- quantile(JH23_females[[log_col]], 0.25, na.rm = TRUE)

  Q3 <- quantile(JH23_females[[log_col]], 0.75, na.rm = TRUE)

  IQR <- Q3 - Q1

  lower_bound <- Q1 - 1.5 * IQR

  upper_bound <- Q3 + 1.5 * IQR

  # Filter data to exclude outliers

  data_filtered <- JH23_females[JH23_females[[log_col]] >= lower_bound & JH23_females[[log_col]] <= upper_bound, ]

  # Filter data to exclude rows with NA values in log_col or Treatment

    data_filtered <- data_filtered[complete.cases(data_filtered[, c(“Treatment”, log_col)]), ]

 plot <- ggplot(data_filtered, aes(x = Treatment, y = !!sym(log_col), fill = Treatment)) +

    geom_boxplot(color = “black”) +

    scale_fill_manual(values = group_colors) +

    labs(x = “Treatment Group”, y = “ln”, title = log_col) +

    theme_minimal() +

    theme(panel.grid.major = element_blank(), panel.grid.minor = element_blank(), axis.line = element_line(color = “black”)) +

    ylim(y_range) +

    guides(fill = FALSE)

  plot_height <- 2

  plot_width <- 2

  print(plot)

    ggsave(paste0(log_col, “_plot.tiff”), plot = plot, width = plot_width, height = plot_height)

}

# Iterate through selected variables and create/print/save plots without outliers

for (log_col in selected_vars) {

  y_range <- c(min(JH23_females[[log_col]], na.rm =

TRUE), max(JH23_females[[log_col]], na.rm = TRUE))  # Adjust the y-axis range as needed

  create_print_save_plots_no_outliers(log_col, plot_width, y_range)

}

```
~~~~

2) Image Augmentation

```
def rotate_image(image):

    rotated_image = cv2.rotate(image, cv2.ROTATE_90_CLOCKWISE)

    return rotated_image

def flip_horizontal(image):

    flipped_image = cv2.flip(image, 1)

    return flipped_image

def flip_vertical(image):

    flipped_image = cv2.flip(image, 0)

    return flipped_image

def add_noise(image, noise_level):

    row, col, ch = image.shape

    mean = 0

    sigma = noise_level

    gauss = cv2.randn(cv2.randn(image.copy(), 0, sigma), 0, sigma)

    noisy_image = cv2.add(image, gauss)

    return noisy_image

# go over the root directory containing class folders

for class_name in os.listdir(root_dir):

    class_dir = os.path.join(root_dir, class_name)

  # checking for directories

    if os.path.isdir(class_dir):

        print(f”Processing images in class folder: {class_name}”)
```

```
    # count original images

 original_image_count = len([filename for filename in os.listdir(class_dir) if filename.endswith((‘.jpg’, ‘.png’, ‘.tif’))])

      print(f”Number of original images in {class_name}: {original_image_count}”)

    # go over files in the class directory

    for filename in os.listdir(class_dir):

      if filename.endswith((‘.jpg’, ‘.png’, ‘.tif’)):

        # load the original image

        original_image_path = os.path.join(class_dir, filename)

        image = cv2.imread(original_image_path)

        if image is None:

          print(f”Error loading image: {original_image_path}”)

          continue

        rotated_image = rotate_image(image)

        flipped_horizontal = flip_horizontal(image)

        flipped_vertical = flip_vertical(image)

        noisy_image = add_noise(image, 30)

        # save the new augmented images

        cv2.imwrite(os.path.join(class_dir, f”rotated_{filename}”), rotated_image)

        cv2.imwrite(os.path.join(class_dir, f”flipped_horizontal_{filename}”), flipped_horizontal)

      cv2.imwrite(os.path.join(class_dir, f”flipped_vertical_{filename}”), flipped_vertical)

    cv2.imwrite(os.path.join(class_dir, f”noisy_{filename}”), noisy_image)

    # count # augmented images

      augmented_image_count = len([filename for filename in os.listdir(class_dir) if filename.startswith((‘rotated_’, ‘flipped_horizontal_’, ‘flipped_vertical_’, ‘noisy_’))])

      print(f”Number of augmented images in {class_name}: {augmented_image_count}”)

print(“Data augmentation completed.”)
```

3) Classification

```
model5 = LinearSVC()

model5.fit(traindata,trainlabels)

guessedlabels5 = model5.predict(testdata)
```

4) Neural Network Automation Model

```
model = Sequential()

model.add(InputLayer(input_shape=[INPUT_SIZE[0],INPUT_SIZE[1],3])) #keras will internally add batch dimension

model.add(Conv2D(filters=32,kernel_size=7,strides=3,padding=’same’, activation=’relu’))

model.add(MaxPool2D(pool_size=3,padding=’same’))

model.add(Dropout(0.3))

model.add(Conv2D(filters=128,kernel_size=5,strides=3,padding=’same’, activation=’relu’))

model.add(MaxPool2D(pool_size=3,padding=’same’))

model.add(Dropout(0.3))

model.add(Conv2D(filters=64,kernel_size=3,strides=1,padding=’same’, activation=’relu’))

model.add(MaxPool2D(pool_size=2,padding=’same’))

model.add(Dropout(0.3))

model.add(Flatten())

model.add(Dense(64,activation=’relu’))

model.add(Dropout(0.3))

model.add(Dense(16,activation=’relu’))

model.add(Dropout(0.3))

model.add(Dense(1,activation=’linear’))

model.compile(loss=’mean_squared_error’, optimizer=Adam(learning_rate=0.001))

model.summary()
```