An Addition to the Toolkit: An Ethogram for *Trichoplusia ni*

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Cover Photograph: Early fifth instar Trichoplusia ni larva from a lab culture at the University of North Georgia photographed on white paper by Margaret Smith.

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An Addition to the Toolkit: An Ethogram for *Trichoplusia ni*

Logan Pearson[1], Chloe Meewes[2], Sydney Spencer[1], Angela Ayala[1], Brad Bailey[3], Erin E. Barding[1], Ryan Shanks[1,*], and Margaret Smith[1]

Abstract - Animal behaviors are a consequence of complex neurobiological mechanisms and environmental interactions and thus can be used to understand these processes. However, we can only study the diversity of animal behaviors in this way if there is a diversity of ethograms across many species. Therefore, we developed an ethogram for *Trichoplusia ni*, the Cabbage Looper, which is well studied in other areas but for which an ethogram did not previously exist. Additionally, we demonstrate the usefulness of this ethogram by manipulating a single environmental variable, the presence of another *T. ni* individual, and demonstrate that some aspects of behavior change while others do not. With the addition of another individual, the type and frequency of behaviors is not altered, but the location where organisms spend time and the distance traveled does change. Therefore, this work rigorously describes *T. ni* behaviors and uses these descriptions to establish a foundation for behavioral studies in this organism and in comparison to others.

Introduction

Behavior can vary among organisms due to a combination of genetic, environmental, learned, social, cultural, neurological, and physiological factors (Gérard et al. 2022, London 2017, York 2018). The complex interplay between these factors contributes to the diversity of behaviors observed among animals. To begin to tease apart the complexities, model organisms in a lab are useful because they can be used to study individual aspects of behavior in a controlled environment. However, to understand the full spectrum of animal behaviors, we must use a variety of models that reflect the diversity of animal life. For example, insect models can be particularly useful to understand the link between neurological changes and clearly defined behaviors (Chen and Hong 2018, Kravitz and Hernandez 2015, McClellan and Montgomery 2023, Steinbeck et al. 2020).

Trichoplusia ni (Hübner) (Cabbage Looper) (Lepidoptera: Noctuidae) has been studied for a variety of reasons, including its role as an agricultural pest and as part of a variety of host-parasite models (Burke and Strand 2014, Gordon and Strand 2009, Kang et al. 1996). Thus it has established research resources, including a commercially available source of material, basic protocols for lab culture, a sequenced genome (Chen et al. 2018), established cell lines (Fu et al. 2018, Maghodia et al. 2020), and well described anatomy and life history (McEwen and Hervey 1960, Shorey et al. 1962). However, currently, there is a paucity of literature describing detailed behavioral analysis of Lepidoptera in a controlled lab setting.

To add to the toolkit available for *T. ni* research, we aim to develop *T. ni* as a behavioral model. As is typical of lepidopterans, *T. ni* has 4 developmental stages (egg, larva, pupa and adult), and thus provides unique and defined developmental windows in which one can study behaviors. Also, they also have well-defined nervous systems that could provide a

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model that allows mechanistic questions to be answered (Caveney and Donly 2002, Fuchs et al. 2014, Gallant et al. 2003, Liu et al. 2023, Malutan et al. 2002, McLean et al. 2005, Tang et al. 2019). Neuroscience investigations can use behavioral alterations as an end-point measurement of change. Development of a comprehensive ethogram that captures the intricate behavioral range of model organisms is a key starting point for these types of studies. To our knowledge, *T. ni* has no comprehensive ethogram established. Therefore, this study establishes a foundational ethogram for *T. ni* larvae and describes its usefulness in a lab setting with a comparison of these behaviors in single and paired animal paradigms.

Materials and Methods

Research population

The research population was taken from a general population of *T. ni* kept in constant culture at 25°C with a 16:8 hr light:dark cycle. Adults were fed a 10% sucrose solution, and all larvae were fed ad lib on cornmeal-based artificial diet (Southland Incorporated). Individuals were separated from the general population in the egg stage and reared in pairs. After hatching, the research individuals in the population were staged every other day, and instar was determined based on head capsule width (McEwen and Hervey 1960). Behavior was observed only in larvae on the first day of their fifth instar, and only one of the pair reared together was used regardless of the behavioral paradigm tested.

Behavioral arena and recording

To document larval behavior, a total of 20 video recordings of a single-animal paradigm (n = 20 individuals) and 20 videos of a paired-animal paradigm (n = 40 individuals) were recorded for 30 min by an HX-WA03 Panasonic camera. The behavioral arena was the bottom of a pipette-tip box with the dimensions 12.5 cm by 10.2 cm (Fig. 1A). The single-animal paradigm was defined as one individual in the arena (Fig. 1B), and the paired-animal paradigm was defined as two individuals in the arena simultaneously (Fig. 1C). In both paradigms, the arena had no food included. Different larvae were recorded for each video. Recordings were stopped at 30 min because it was empirically determined that no new behaviors were exhibited after 30 min. Dark purple cardstock was placed underneath the arena to prevent glare as the recordings were taken inside the incubator during the light cycle. The camera was suspended above the arena during recording, and the distance between the camera and the arena was approximately 30 cm. The arena floor was sanded to roughen the plastic surface. The walls of the arena were not sanded, and petroleum jelly was applied along the top edges using a sterile cotton swab to prevent the individuals from crawling out of the arena. In addition, the pipette box was cleaned with ethanol between each use to prevent any residual cues from previous trials.

Depending on the behavioral paradigm being recorded, the larvae were placed in different areas of the arena (Fig. 1B and C). For the single-animal behavioral paradigm, the larva was placed in the center of the arena at the beginning of the recording (Fig. 1B). For the paired-animal paradigm, each larva was initially placed in opposing quadrants known as "native" for each animal (digitally labeled A and B in Fig. 1C and D). The quadrant of the opposing animal was termed "non-native" for each animal. There were also 2 other quadrants labeled "neutral", where no organism was initially placed. For both the single-animal and paired-animal paradigm, the quadrant in which the animals spent the most time was referred to as the "primary" quadrant (Fig. 1B and D).

Ethogram development

The examination of behavior took place once the recordings were taken, with independent coders coding each video. Collaboratively, the coders developed a list of behaviors and their associated descriptions for each paradigm. This baseline ethogram was used to independently record the frequency of each behavior exhibited in 5-min bins over 30 min. Extending the time beyond 30 min did not reveal additional behaviors or unique frequencies.

Two coders watched videos independently and named, defined, and documented behaviors exhibited by the individual larva (Table 1, Supplemental Data). The coders then compiled their list of behaviors into a master list that was used to code several more videos. To test the quality of the definitions, the list of behaviors was given to additional coders who were not directly involved in the development of the ethogram to independently identify behaviors. All behaviors unable to be accurately coded were redefined in the ethogram. This process was iterated until there was an average intraclass correlation coefficient (ICC) of 0.89 between new coders.

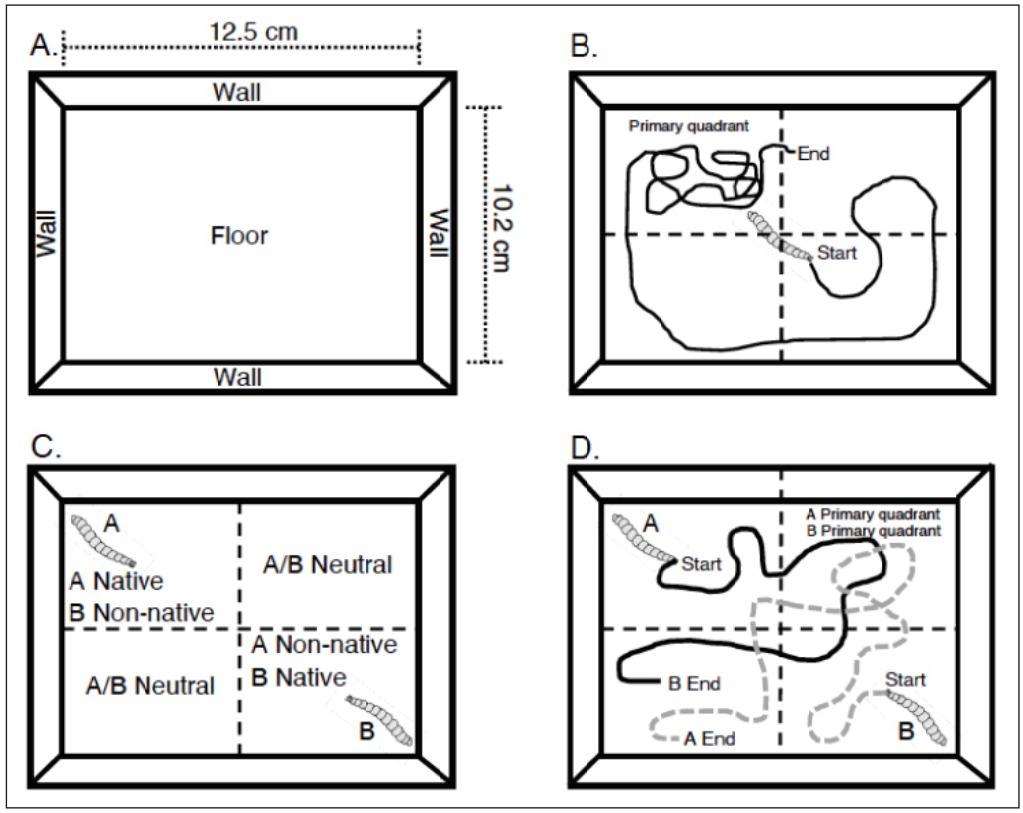

Figure 1. Behavioral arena for coding behavior for single (one animal) and paired (two animals) paradigms. (A) Arena dimensions for all behavioral experiments. (B) The single behavioral paradigm started with the larva in the center of the arena. Distance and time were measured for each quadrant with the primary quadrant representing the one with the greatest value for distance and time. (C) The paired behavioral paradigm started with the larvae (A and B) in opposing quadrants. The starting quadrant for A (native) is the non-native quadrant for B and visa-versa. Quadrants where no larva started were considered neutral. (D) Distance and time were measured for native, non-native, and neutral quadrants. The primary quadrant represents the one quadrant with the greatest value for distance and time for each larva individually.

Table 1. *Trichoplusia ni* ethogram

Type of behavior	Behavior	Description	Visual aid
Locomotion	Inchworm	Continuous movement whereby the individual's prolegs and true legs remain suctioned to the surface and slide toward and away from each other while its body arches upward and stretches out. Additionally, any range of that arch is considered inchworm behavior. (See Supplemental File 1, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s1.mp4).	
	Vertical movement	Individual latches both its true legs and prolegs onto the petroleum jelly covered the edges of the arena. (See Supplemental File 2, available online at http:https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s2.mp4).	
	Still	Individual exhibits no movement; it is still for at least 5s. (See supplemental File 3, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s3.mp4).	
Elimination	Frass	Excretion of feces while the individual is in the arena; larval frass is circular and light brown in color. If the individual releases 1 pellet, record that as 1 frass behavior. (See Supplemental File 4, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s4.mp4).	
	Emesis	Individual expels fluid from mouth; larval vomit is a green fluid-like substance. (See Supplemental File 5, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s5.mp4).	
Non-Locomotion	Head movement	Individual's prolegs and true legs remain attached to the surface of the arena while only its head moves (the area from the tip of its head to the area right before its true legs). (See Supplemental File 6, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s6.mp4).	 
	Upward extension	The individual's prolegs are suctioned to the surface of the arena and it brings its anterior body upward where it holds a still position as seen in the picture. Individual remains still in this position for longer than 1 second. (See Supplemental File 7, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s7.mp4).	
	Searching	Individual's prolegs suction to the ground while its thorax extends upward and moves from one direction to another (can move L-R or R-L); during this movement, the individual's true legs are not attached to the surface of the arena. (See Supplemental File 8, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s8.mp4).	 
Engagement	Wall engagement	An individual engages with the walls of the arena by latching its true legs onto the petroleum jelly covered surface and maintaining its proleg grip on the bottom surface of the arena. (See Supplemental File 9, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s9.mp4).	
	Body engagement	Individuals engage with its body by keeping prolegs on the surface and bringing head and true legs backwards/forwards to a part of its body. (See Supplemental File 10, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s10.mp4).	
	Frass interaction	Individuals interact with their frass by nudging their frass with their head. (See Supplemental File 11, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s11.mp4).	
Sociability	1-Sided interaction	Any part of individual A or B contacts any body part of individual A or B. (See Supplemental File 12, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s12.mp4).	
	2-Sided interaction	Individual A or B physically contacts the other individual and this elicits a response whereby the other individual uses its head to push A or B away from itself. (See Supplemental File 13, available online at https://eaglehill.us/ebioonline/suppl-files/ebio-036-Shanks-s13.mp4).	

Behavioral coding

Once the ethogram was established with strong inter-relater reliability, new coders analyzed 30-min recordings of the individual and paired behavioral paradigms. They were provided with the established ethogram, to which they had not been previously exposed. The coders then tallied each time a behavior was displayed according to the terms and definitions they were given. All behaviors were coded in 5-min bins.

Time and locomotion

To further understand the locomotion patterns of the larvae studied in both paradigms, the arena was divided into quadrants (Fig. 1). The time spent and total distance traveled in each quadrant were measured. The locomotion distance was measured with a ruler by tracing the movement of the individual using a sheet of transparency paper, and the amount of time spent in each quadrant was tracked for each 5-min bin (Fig. 1B and D).

Data analysis

For tallies of individual behaviors, high-occurrence behaviors were defined as those exhibited 10 or more times in a 30-min recording. Low occurrence behaviors were defined as those exhibited less than 10 times in a 30-min recording. Independent sample t-tests were performed to determine if the frequency of high-occurrence behaviors differed between single-animal and paired-animal paradigms. Similarly, time and distance traveled in different quadrants, and frequency of one-sided vs two-sided interactions were analyzed with t-tests. Duration of time spent in the primary quadrant across time intervals and paired paradigms were analyzed with ANOVA. Statistical analyses were done in R (v. 4.2.2) and SPSS (v. 29.0). In all graphs, error bars represent the standard error (SE).

Results

Ethogram

To develop this ethogram for *T. ni*, we collected behavioral data from 20 single-animal videos and 20 paired-animal videos. We only recorded the larvae in the first day of the fifth instar for consistency and visual purposes (Table 1, Supplemental Data).

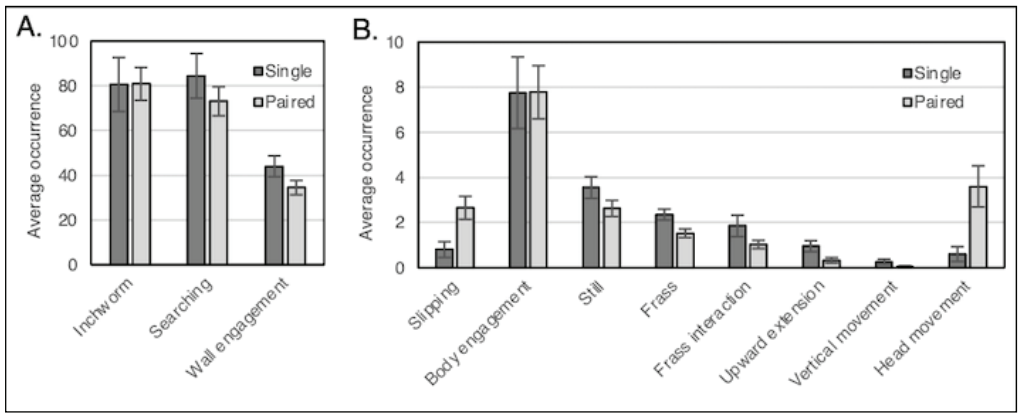

Figure 2. Coded larval behaviors defined in the ethogram measured over 30 minutes for both the single (dark columns) and paired (light columns) behavioral paradigms. (A) High-occurrence behaviors (B) Low-occurrence behaviors. Error bars represent ± standard error (SE).

Behavior

Using the ethogram, coders tallied behaviors exhibited during 30-min recordings. We were able to distinguish the high-occurrence behaviors (Fig. 2A) from low-occurrence behaviors (Fig. 2B). There was no statistical difference in the frequency of high-occurrence behaviors between single-animal and paired-animal paradigms (inchworm: $t_{58} = 0.02$, $P = 0.98$; searching: $t_{58} = 0.98$, $P = 0.33$; wall engagement: $t_{58} = 1.70$, $P = 0.09$). The scarcity of low-occurrence behaviors did not warrant statistical analysis of single-animal and paired animal paradigms (Fig. 2B).

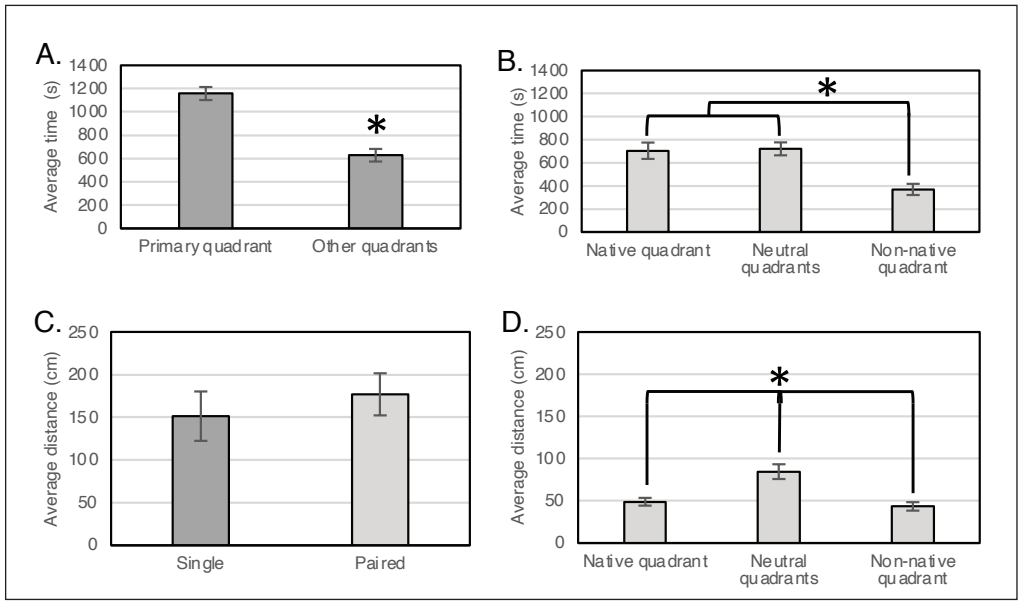

Figure 3. Coded larval average time (A and B) and average distance traveled (C and D) in 30 minutes for both the single (dark columns) and paired (light columns) behavioral paradigms. * indicates $P < 0.001$. Error bars represent ± SE.

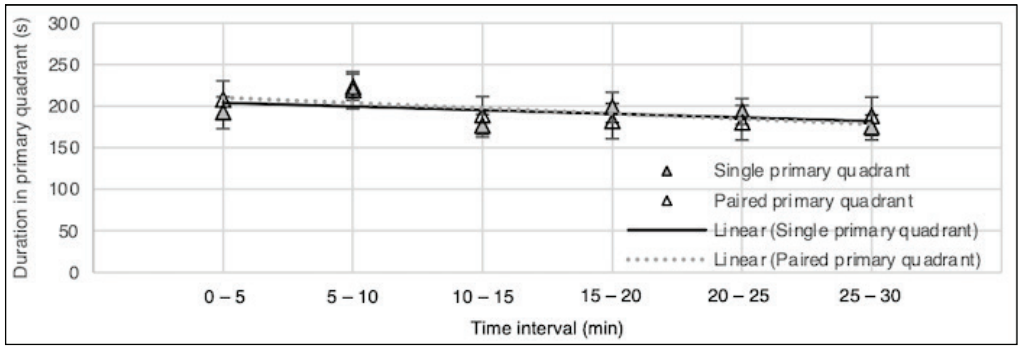

Figure 4. Average time spent in the primary quadrant for larvae in both the single (dark triangle and solid linear trend line) and paired behavioral paradigms (light triangles and dashed linear trend line) shown in 5-minute time intervals for the 30-minute coding period. Error bars represent ± SE.

Time and distance

Time and distance traveled in different quadrants was measured for both single-animal and paired-animal paradigms. The single-animal paradigm revealed more time spent in 1 quadrant compared to the other 3 quadrants (Fig. 3A, $t_{38} = 6.8$, $P < 0.001$). The paired-animal paradigm also showed that there was an effect of quadrant on time spent in the quadrant (Fig. 3B, $F_{(2,117)} = 11.26$, $P < 0.001$). A post-hoc Tukey's test indicated that individuals spent less time in the non-native ($P < 0.001$) quadrant than either the native or neutral quadrants.

There was no statistical difference in the total distance traveled by individuals in the single-animal paradigm versus paired-animal paradigm (Fig. 3C, $t_{58} = 0.89$, $P = 0.37$). In contrast, for the paired-animal paradigm, (Fig. 3D), there was a significant difference in the average distance traveled in different quadrants ($F_{(2,117)} = 12.23$, $P < 0.001$). A post-hoc Tukey's test indicated that individuals spent significantly more time in neutral quadrants than either their native quadrant ($P < 0.001$) or non-native quadrant ($P < 0.001$). This increase is attributed to the fact there is twice as much space in the neutral quadrants, yet remains notable given the decreased time spent in the non-native quadrant only.

Time interval *vs* duration in primary quadrant

The duration of time spent in the primary quadrant across time intervals was also examined (Fig. 4). For the single-animal paradigm, there was no significant difference between the 5-min time intervals and time spent in a primary quadrant versus other quadrants as indicated by a regression line slope not statistically significantly distinguishable from 0 ($F_{(5)} = 0.921$, $P = 0.339$). In contrast, for the paired-animal paradigm, individuals decreased the amount of time they spent in their primary quadrant over time as indicated by a statistically significant negative slope of the regression line ($F_{(5)} = 5.81$, $P = 0.017$).

Interactions

Lastly, 2 novel behaviors (one-sided interactions and two-sided interactions) occurred only in the paired-animal paradigm recordings (Table 1, Fig. 5). One-sided interactions were significantly more frequent than two-sided interactions ($t_{78} = 2.77$, $P = 0.007$).

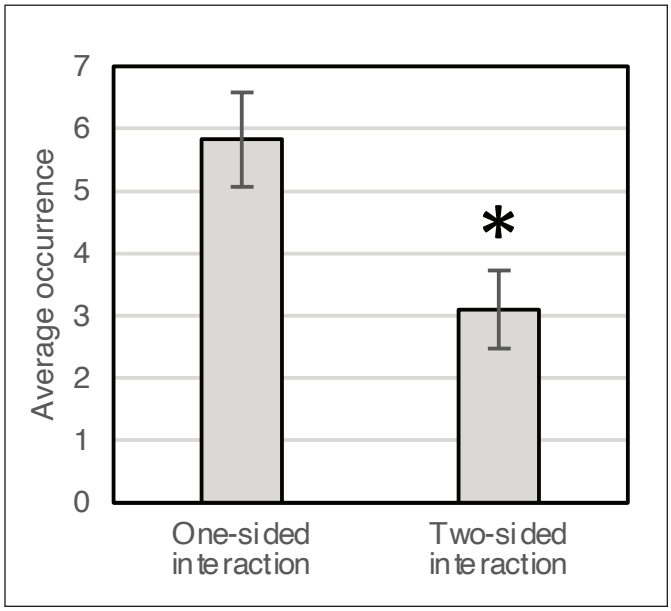

Figure 5. One-sided and two-sided interactions measured between larvae in the paired-animal paradigm over 30 minutes. * indicates $P < 0.05$. Error bars represent ± SE.

Discussion

Ethogram

Establishing a well-defined ethogram is fundamental to understanding animal behavior (Okuyama et al. 2013). With clear operational definitions, this ethogram was created to give other researchers the opportunity to use a straightforward system to answer additional questions (Xu et al. 2012). In addition to these behaviors, methodology for how to track and time the movement of animals within a defined arena provide a template for future studies (Fig. 1, Table 1, Supplemental Data). Whilst many researchers have investigated specific behaviors of interest, a more inclusive description of behaviors allows for a more integrative and cross-disciplinary approach (Collie et al. 2020, McLellan and Montgomery 2023, Okuyama et al. 2013, Peric-Mataruga et al. 2017, Suszczynska et al. 2017).

Behavior

To illustrate the application of the operational definitions in the ethogram, the behaviors were coded with the addition of a single variable that compares a single larva's behaviors alone and with an additional larva. High occurrence behaviors show no statistical difference with this single environmental variable we exampled (single *vs.* paired), yet this may provide an interesting comparison to the effect of different environmental variables such as food availability and temperature (Fig. 2A). Similarly, while low frequency behaviors hold no validity statistically due to the low number of coded events, there were trending differences that may hold biological meaning (Fig. 2B). Therefore, the importance of these recorded events lies beyond any statistical findings. This ethogram and the example data analysis clearly defined all possible behaviors so that future studies may alter environmental, social, or developmental variables or compare behaviors between species in a more wholistic approach. For example, the upward extension of the head provided little measurable difference between coded behaviors within either the single or paired behavioral paradigms, yet this behavior may be drastically altered if food were introduced into a competitive environment in which two animals shared the arena. However, for the ability to conduct future comparative and meta-analysis, it will be important that all behaviors regardless of their statistical significance be documented.

Location and distance

As with behavior, measures of location and distance traveled provide an important measurement of neurobiobehavioral alterations. This can be used to measure responses to the introduction or removal of environmental cues and gauge whether they are appetitive or non-appetitive. Individuals in both the single and paired behavioral paradigms had unique location and distance patterns of behavior. These patterns of behavior provide a unique baseline data set to investigate alterations in these behaviors including investigations of motivation, competition, and social interactions. For example, the addition of frass or a potential food source in one area of the arena may alter the primary quadrant for an individual in a single behavioral paradigm. In a paired behavioral paradigm these additions may alter the duration that individuals display in a primary quadrant over time (Fig. 4). Interestingly, the presence of an additional organism did not impact the distance traveled by individuals, yet individuals in both the single and paired behavioral paradigms did have unique location and locomotion patterns (Fig. 3). Individuals in the paired behavioral paradigm did not travel longer distances in their native quadrant; however, they spent more time in the native quadrant (Fig. 3). The added individual B in the paired para-

digm could help explain why individual A spent more time in the native quadrant whilst at the same time traveled less in said quadrant (Fig. 1 and 4). Therefore, it is the combination of both time spent and distance traveled together that is important to consider. Additionally, by binning the duration spent in specific quadrants, unique trends were observed in the individuals of the single and paired behavioral paradigms (Fig. 4). Manipulation of this simple variable (paired *vs.* single) alters behavior in some measurable and meaningful ways (location, Fig. 3) yet has little effect on other behaviors (high-occurrence, Fig. 2). This highlights the usefulness of establishing an ethogram using a controlled laboratory environment in which an arena of a defined size is used.

Interaction

Individuals in the paired-animal paradigm exhibited seemingly aggressive one-sided and two-sided interactions. This is not unusual, even to the point of cannibalism, in both eusocial and solitary insects where its function is used to defend territories, establish social hierarchies, and compete for food (Bowen et al. 2008, Collie et al. 2020, Dial and Adler, 1990, Kemp 2000, Semlitsch and West 1988, Tang et al. 2016, Yack et al. 2001, Zago-Braga and Zucoloto 2004, Zhou et al. 2016). While *T. ni* is not a eusocial insect, it can live in groups, like other lepidopterans, and this may impact behavior (Daly et al. 2012). Figure 5 documents significantly more occurrences of one-sided interactions compared to two-sided interactions in the paired-animal behavioral paradigm. The role these interactions have in the establishment of a potential social hierarchy in *T. ni* remains unclear, but this foundational data opens the door to investigations of how size, developmental stage, and fitness relate to the establishment of potential dominant and submissive individuals in a population. Furthermore, these investigations relate to more broad questions of how social interactions lead to the evolution of important survival behaviors in a group setting.

The combined study of interactions and other behaviors in this ethogram serves as a valuable tool for researchers to further investigate the ecological, physiological, and behavioral aspects of *T. ni*, aiding in the understanding of its biology and ecology. It could open the door to investigations of more broad behavioral notions such as learned helplessness, usually studied in vertebrate models (Maier and Seligman 2017), or other cued social interactions that usually depend on multiple sensory inputs in insects as well as the underlying neural mechanisms dictating these behaviors (Chen and Hong 2018). The analysis of behavioral data in this ethogram can be used as a reference of a standard social interaction amongst *T.ni* and instigate the study of the underlying neural mechanisms causing these interactions to occur. Understanding the neural basis of *T. ni* behavior can shed light on the neural circuits, sensory processing, and neurophysiological processes involved in not only insect but more complex animal behavior. This research provides a foundation for future studies on *T. ni* and other lepidopteran species, with the potential to uncover novel insights into the neural mechanisms underlying insect behavior and inspire future research in neuroethology.

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