

Increased aggression among *Asynarchus nigriculus* caddisfly larvae in a rapidly drying environment

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Abstract:

Global climate change will likely result in warmer, dryer environments and influence the behavior and development of a number of species. *Asynarchus nigrificulus* is a species of caddisfly whose larval stage lives exclusively in temporary ponds where they develop synchronously and quickly before the ponds dry in the early summer. The cues used by this species to sense pond drying and accelerate development are unknown but may include increasing density due to shrinking pond area and decreasing water level. It has been shown that *A. nigrificulus* engage in cannibalism to supplement their nutrient-poor diet of detritus with protein. I hypothesized that if decreasing water level and increased density are cues of impending pond drying, then caddisfly larvae will respond to those cues by engaging in more cannibalism in order to obtain more protein to develop faster and emerge before the pond dries. Field experiments were set up varying two cues of drying: water level and larval density, and also manipulating supplemental protein. Development time and larval behaviors were compared statistically among treatments. The results demonstrated that aggressive interactions among larvae, and escalations of aggressive behavior were highest in the drying treatment without protein supplementation, and the fewest number of larvae pupated in that treatment. Aggressions, escalations, as well as mobbing of one larva by others, also increased with increasing larval density. The frequency of cannibalism was low and inadequate for comparisons between treatments; however, the other aggressive behaviors are precursors to cannibalism. This species, like many, will need to respond to cues associated with a drying environment, and may need to alter its development to persist in the face of climate change. This study aids in the understanding of how aquatic invertebrates and other animals respond to ongoing environmental changes.

Introduction:

Global climate change has been forecast to result in a dryer, warmer climate in many parts of the world. Global temperatures are projected to rise between 1.8 and 4.0° C by end of this century, and the western mountainous regions of the United States are predicted to become drier due to decreased precipitation (IPCC 2007). This changing climate will create new stressors for many organisms and force either phenotypic plasticity within a generation, adaptation over time, or extinction.

The Mexican Cut Nature Preserve near Gothic, Colorado (elevation 3400m) includes many permanent and temporary ponds that contain a number of aquatic invertebrate species adapted to different biotic and abiotic conditions (Batzner and Wissinger 1996). It is an excellent environment to test consequences of climate change because of the diverse ponds sensitive to abiotic stressors. Cased caddisfly species have been shown to exhibit a vast array of tradeoffs in life histories across permanence gradients within the aquatic habitats (Wissinger et al. 2003).

During summer 2012 we witnessed dramatic phenological changes in the hydrology of temporary ponds of the Mexican Cut, which affected aquatic invertebrates that must complete their larval stage in water. The number of days the temporary ponds were filled was shorter than previous years due to snowpack melting early and a lack of precipitation (Wissinger, unpublished data 2012). *Asynarchus nigriculus* is the dominant Limnephilidae caddisfly species in temporary ponds of the Mexican Cut (Greig and Wissinger 2010). Its distribution in ponds that dry in early summer reflects the species' rapid synchronous development and minimal investment in antipredator defense (Wissinger 2004).

Several temporary ponds dried before *A. nigriculus* larvae could develop and emerge. Evidence of larvae dying from desiccation suggests selection is ongoing for fast development in

this environment (Greig and Wissinger 2010). *A. nigriculus* pass through five fixed instars but their development has been known to speed up when faced with pond drying, unlike other caddisfly species (Wissinger et al. 2003). However, the exact cues used by this species to develop quickly in the face of imminent drying are unknown. These cues may include decreasing water levels and increasing larval density as the surface area of the pond bottom decreases.

The diet of *A. nigriculus* larvae consists mostly of nutrient-poor detritus within these oligotrophic habitats. Cannibalism via mobbing of a single individual is a unique behavioral trait of *A. nigriculus*, which provides the larvae with protein not available in the detritus diet (Wissinger et al. 2004). Cannibalizing a conspecific of the same size has its risks, including injury or becoming a secondary victim, but there are clear benefits. The protein obtained via cannibalism has been experimentally shown to speed up development and emergence time, as well as affect adult fitness because adult caddisflies do not feed (Wissinger et al. 2004).

To evaluate how *A. nigriculus* responds to a rapidly drying environment, we simulated pond drying using two cues: decreasing water level, and increasing larval density in two separate experiments. In the first experiment we varied water level and also protein supplementation, and the second varied area and thereby density of larvae in simulated microcosm ponds. Measured responses for both experiments included development time, the incidence of cannibalistic mobs, as well as different aggressive behaviors that act as precursors to cannibalism.

I hypothesized that if decreasing pond bottom surface area, linked to increasing density, and decreasing water level are signals used by *A. nigriculus* larvae to sense pond drying, then cannibalism would increase in response to those cues as a means of obtaining the protein necessary to develop faster and emerge from the pond to avoid desiccation. In addition to the environmental changes, protein supplementation was an independent variable used to test

whether cannibalism, as a means for obtaining protein, or other aggressive behaviors that may be precursors of cannibalism, would decrease when another animal protein source was available.

Additionally, I predicted that the fastest development time and the most aggressive behavior would occur in the decreased water level and increased density treatments because the caddisfly larvae were pressured to emerge faster. Understanding how *A. nigriculus* responds to environmental change and accelerates their development is important for this species and other aquatic invertebrates whose development depends on aquatic resources in a changing climate.

Methods:

Drying Simulated with Decreasing Water Level

We conducted an interactive experiment over 14 days to evaluate the effects of decreasing water level and protein supplementation on behavior and development time of *A. nigriculus* larvae. Sixteen microcosms comprised of 0.25 m² rectangular plastic containers were used with four replicates of each experimental treatment: with and without protein supplement crossed with constant and decreasing water levels. To simulate the natural temporary pond environment, the containers were filled with 60(±10) g of mixed pine/fir detritus and 14.7(±2.4) g of mixed sedge/grass detritus, dry weight. Two rocks and one branch were added as well to provide substrates for pupation. Approximately 75 cm² of gravel, including fine pebbles, were added so the larvae had access to material to prepare pupal cases. Fifty fourth-instar *A. nigriculus* larvae with an average dry weight of 4.41mg were added to each container, for a density of 200m², which is comparable to densities sampled the same year in temporary ponds. Caddisfly larvae were collected from drying temporary ponds in which the larvae were not likely to complete development before the ponds dried.

Protein supplementation consisted of $684.6(\pm 11.7)$ mg of freeze-dried *Tubifex* worms (Wardley brand *Tubifex*: minimum crude protein 50%, crude fat 5%) distributed evenly onto detrital material once weekly in each experimental container. The replicates with no drying treatment were kept at a constant water level of 11cm. Drying was simulated from the first day with the water level beginning at 7 cm and decreased gradually thereafter (Figure 1). The drying treatment water level began lower than 11cm because caddisfly development and pupation was rapidly approaching. On several days it rained and the water level increased slightly. Any deviations from the established water level heights were adjusted by adding water from nearby permanent ponds or by withdrawing water.

Aggressive behavior was measured using methods adapted from Wissinger et al. 1996. Behavioral observations were conducted in each microcosm for five minutes, every other day. Measured behaviors are listed in Table 1 and include time active, the total numbers of interactions, those that were aggressive, the proportion aggressive, those that escalated, and the proportion that escalated were recorded. The proportion aggressive was calculated by dividing aggressive interactions by total interactions observed and the proportion that escalated was calculated by dividing escalations by the number of aggressive interactions. These behavioral measures were used in addition to total aggressions and escalations because the number of total interactions and aggressions varied between replicates.

All cannibalistic mob events and explicit cannibalism observed were also recorded. Mob events were identified as a group of at least 5 individuals with their heads in the middle of the group. Cannibalism was more difficult to distinguish because it required the victim to be visible within the cannibalistic mob.

Two different kinds of behavioral observations were implemented, 5-minute focal observations and 5-minute scans, which were used for different reasons to provide a comprehensive evaluation of caddisfly larvae behavior. The focal observations followed a single larva, which provided more precision; however this method could result in a bias toward selection of the most active individuals for observation. Scanning, on the other hand, takes individual variation into account by observing all larvae in the microcosm over a 5-minute period. However, the precision is sacrificed because it is difficult to record all behaviors that take place in a microcosm and to avoid recording events more than once.

Rock and tree substrate in the containers were inspected daily for pupae, which were promptly removed and placed in pupation chambers where emergence could be closely monitored. For unknown reasons no pupae emerged as adults; all of the pupae died before emerging. Therefore the measure of development analyzed in this study was only the time from the fourth-instar to pupation.

Drying Simulated with Decreasing Area

A second experiment was conducted over 12 days to measure the effect of increased larval density as a cue for pond drying on development time and behavior. Three replicates of four plastic container sizes each contained 153 caddisfly larvae. The bottom surface areas (and densities) were as follows: 1.65 m² (93 larvae/m²), 0.79m² (193 larvae/m²), 0.25 m² (612 larvae/m²) and 0.051 m² (3000 larvae/m²). These increasing densities are comparable to those observed in the field when the habitat available in a temporary pond decreases a hundred-fold in size while drying and the density reaches thousands of larvae per meter squared (Personal Observation and Image 1).

Fourth and fifth instar caddisfly larvae for this experiment, with an average dry weight of 6.9mg, were gathered from drying temporary ponds. Gravel, rocks, underwater branches, and 1cm of detrital material were added to each tank and the water level was kept constant. The larvae in the smallest containers died overnight on Day 3, likely of anoxia. Thereafter we kept those larvae in a separate holding tank and distributed them into the smallest containers at least one hour before behavioral observation, then returned them to the large tank. Because those larvae were not kept in a consistently high-density environment, we did not collect pupation data for the 3000 larvae/m² density treatment.

Similarly to the first experiment, focal animal and scanning behavioral observations were made for this experiment until most of the larvae pupated. Development times were recorded, although, for unknown reasons there was only one pupa that emerged as an adult, while the rest died without emerging.

Statistical Analysis

The behavioral data obtained from both experiments were analyzed using ANOVA tests and post-hoc t-tests for pairwise comparisons. Where appropriate, interactions between treatments were analyzed to evaluate the hypotheses for each measured variable. The independent variables were water level and protein supplementation for the first experiment, which were analyzed with a 2-way ANOVA. Larval density was the independent variable in the second experiment and was evaluated using a 1-way ANOVA. The dependent variables included time active, multiple behaviors, as well as incidence of mobbing and cannibalism. Differences in development times between treatments were analyzed for both experiments using a chi-square test to compare the observed number of pupae collected for each treatment to values expected by random chance. Pupation rates were also compared between treatments for each experiment

using a cumulative relative frequency plot and Kolmogorov-Smirnov tests. All of the above tests were performed using R statistical software (R Core Team 2012).

Results:

Across both experiments, the scanning and focal observations showed very consistent trends for each measured behavior, although the specifics of significant differences varied among observed behaviors. Therefore both kinds of observation reasonably described the responses by caddisfly larvae to the treatments.

Drying Simulated with Decreasing Water Level

Behavior

We observed a range of behavioral responses to the drying and protein treatments. Interactions and escalations recorded under the focal observations were natural log transformed before analysis in order to better fit the assumptions of distribution for the ANOVA test. $\ln(\text{Interaction})$ and $\ln(\text{Escalation})$ were found to be significantly dependent on protein and drying treatments, respectively (ANOVA, Table 2).

A. nigriculus exposed to drying treatments were significantly more aggressive in both the focal and scanning observations (Table 2). The focal observations also revealed a decrease in aggression when protein was added. The effect of protein supplementation on the number of aggressive behaviors observed did not vary with drying treatment (no significant drying $\times$ protein interaction; ANOVA, $p=0.217710$, $F=1.535$, $df=1$). However, pairwise comparisons showed that the highest rate of aggressive interactions occurred in the drying treatments with no protein added (Figure 2).

The percent of aggressive interactions observed during focal observation was also found to be dependent on drying but was the only variable in which the effect of protein

supplementation varied with drying treatments as shown in Figure 3. Escalating aggressive behavior observed during scans was an additional behavior that varied significantly with drying treatment (Table 2).

There were only five mobbing events and no explicit cannibalism observed in all observations of the first experiment, so no formal analysis was performed on those variables. However, all observed mobbing occurred in drying treatments.

Pupation

A total of 154 pupae were recovered in the microcosms with the following distribution among the four treatments: 22 (+drying+protein), 11 (+drying-protein), 57 (-drying+protein), and 64 (-drying-protein). A chi-square test revealed that the results were statistically different than expected by random chance ($\chi^2=52.494$, $df=3$, two-tailed p -value <0.0001). The deviation from random distribution of pupation can be attributed to significantly fewer individuals pupating in the drying treatments than the non-drying treatments (Figure 4), with no apparent effect of protein supplementation on the number of pupae observed. A two-sample Kolmogorov-Smirnov test showed that the pupation rate did not significantly differ between drying treatments (K-S test; $D=0.2308$, two-sided $p=0.8793$).

Drying Simulated with Increasing Density

Behavior

Most of the measured behavioral variables in the second experiment significantly increased in occurrence with an increase in larval density (ANOVA, Table 3). For example, in the scanning observations, the number of aggressive interactions (Figure 5), the number of escalations (Figure 6), and mobbing behavior (Figure 7) increased with larval density and the

relationships between those behaviors and natural log-transformed larval density were fit best to a 2nd order polynomial linear regression with an intercept at (0,0).

Within the focal observations, the effect of density on the number of overall interactions and aggressive interactions varied with the date of observation (2-way ANOVA; Interactions: Density $\times$ Julian Date, $F=4.874$, $p=0.0307$, $df=1$. Aggression: Density $\times$ Julian Date, $F=6.026$, $p=0.01670$, $df=1$). Both total interactions and aggressive interactions decreased over the course of the experiment in the focal observations. Due to the interaction between density treatment and date of observation, these dependent variables were not included in the final analysis.

Pupation

A total of 323 pupae were recovered in the tanks with the following distribution among the treatments: 45 (93 larvae/m²), 84 (193 larvae/m²), and 194 (612 larvae/m²). The highest density treatments did not yield any pupae because the larvae could not survive in such conditions and were therefore placed in high-density treatments only for behavioral observation. A chi-square test revealed that the distribution of a higher number of pupating larvae in higher density treatments was statistically different than expected by random chance ($\chi^2=111.015$, $df=2$, two-tailed p -value <0.0001). Figure 8 shows the accumulation of pupae over time for each density treatment. A cumulative relative frequency plot and one-sided Kolmogorov-Smirnov tests between each density showed that the pupation rate did not significantly differ between density treatments (One-sided K-S tests; 93 and 193 larvae/m²: $D=0.375$, p -value=0.3247, 93 and 612 larvae/m²: $D=0.375$, p -value=0.3247, 193 and 612 larvae/m²: $D=0.5$, p -value=0.135).

Discussion:

Due to their temporary aquatic habitat, *Asynarchus nigriculus* caddisfly larvae must be able to respond to environmental drying cues in order to survive, especially in a future drier

environment resulting from global climate change. This study found that *A. nigriculus* are able to sense and respond to both decreasing water level and increasing larval density as cues of pond drying with aggressive behavioral changes indicating an increase in cannibalism. No significant differences in pupation rates were found between treatments, but there were significantly different numbers of pupae between treatments.

Drying Simulated with Decreasing Water Level

Observed behaviors including total interactions, aggression, and escalation were all highest among larvae in the drying treatment without protein supplementation. While we did not observe cannibalism in the experiment, these behaviors could be considered precursors to, and indications of cannibalism. Significantly higher aggression in the drying treatment without protein supplementation compared to that with protein supplementation suggests that aggression, and ultimately cannibalism, is a means to obtain more protein and confirms research by Wissinger et al. (2004). These behavioral observations are consistent with the hypothesis that *A. nigriculus* responds to decreased water level, a signal of pond drying, by acting more aggressively as precursor to cannibalism in order to obtain enough protein to complete development.

There were significantly fewer pupae found in the drying treatments compared to the non-drying treatments, which directly opposes the prediction that larvae will develop faster when stressed with drying (Wissinger et al. 2003). This result may be due to other environmental conditions, potentially altered water temperatures. The drying treatments, containing much less water than the non-drying treatments, experienced more extreme temperature highs and lows. The range of temperatures was, on average, 2.8° C greater in drying treatments than non-drying treatments, in higher and lower extremes. The nearby semi-permanent pond experienced even

smaller daily temperature fluctuation. These temperature extremes experienced in exposed plastic containers with low water level could have stunted the development of the caddisfly larvae. The optimum development temperature for *A. nigriculus* caddisfly larvae is currently unknown.

Another aquatic invertebrate, the stonefly nymph, was found to increase its growth rate with increasing temperature in subalpine environments (Brittain 1983). Approximately 90% of the variation in growth rates could be explained by temperature differences (Brittain 1983). Sweeney and Vannote also found that temperatures correlated with observed differences in larval growth rates of stream stoneflies (1986). Clearly temperature has a strong influence on the development and growth of aquatic invertebrates.

Drying Simulated with Increasing Density

Using density to simulate pond drying resulted in significant increases in aggressive interactions among caddisfly larvae with increased density, including mobbing. The number of larvae pupating also increased significantly with increased larval density. This outcome is consistent with our predictions, that as density increases, larvae become more aggressive, engage in more cannibalistic mobs, and develop in order to emerge faster, thereby avoiding desiccation.

In this study, we found that *A. nigriculus* responds to the environmental signals of decreased water level and increased density by becoming more aggressive, which leads to increased cannibalism. This behavioral modification in the face of environmental drying stress provides insight into how animals respond to changing climates and has implications for aquatic invertebrate development in a future climate that is warmer and drier.

-Need to relate to other studies

Future research will include repeating the first experiment, varying water level and protein supplementation, but keeping the water temperature consistent between treatments using precise electrical heating and cooling. It would also be useful to find what temperature is most advantageous for caddisfly larvae development because warming due to climate change could have implications for development rates based on water temperature.

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Literature Cited:

1. Batzer, D.P., S.A. Wissinger. 1996. Ecology of insect communities in nontidal wetlands. *Annual Review of Entomology* 41: 75-100.
2. Brittain, J.E. 1983. The influence of temperature on nymphal growth rates in mountain stoneflies (Plecoptera). *Ecology* 64 (3): 440-446.
3. Greig, H.S. and S.A. Wissinger. 2010. Reinforcing abiotic and biotic time constraints facilitate the broad distribution of a generalist with fixed traits. *Ecology* 91 (3): 836-846.
4. Intergovernmental Panel on Climate Change. 2007. Fourth Assessment Report.
5. R Core Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL <http://www.R-project.org/>.

6. Sweeney, B.W. and Vannote, R.L. 1986. Growth and production of a stream stonefly: influences of diet and temperature. *Ecology* 67 (5): 1396-1410.
7. Wissinger, S.A., W.S. Brown, and J.E. Jannot. 2003. Caddisfly life histories along permanence gradients in high-altitude wetlands in Colorado (USA). *Freshwater Biology* 48: 255-270.
8. Wissinger, S.A., J. Steinmetz, J.S. Alexander, and W.S. Brown. 2004. Larval cannibalism, time constraints, and adult fitness in caddisflies that inhabit temporary wetlands. *Oecologia* 138(1): 39-47.
9. Wissinger, S.A., G.B. Sparks, G.L. Rouse, W.S. Brown, and H. Stelzer. 1996. Intraguild predation and cannibalism among larvae of detritivorous caddisflies in subalpine wetlands. *Ecology* 77: 2421-2430.

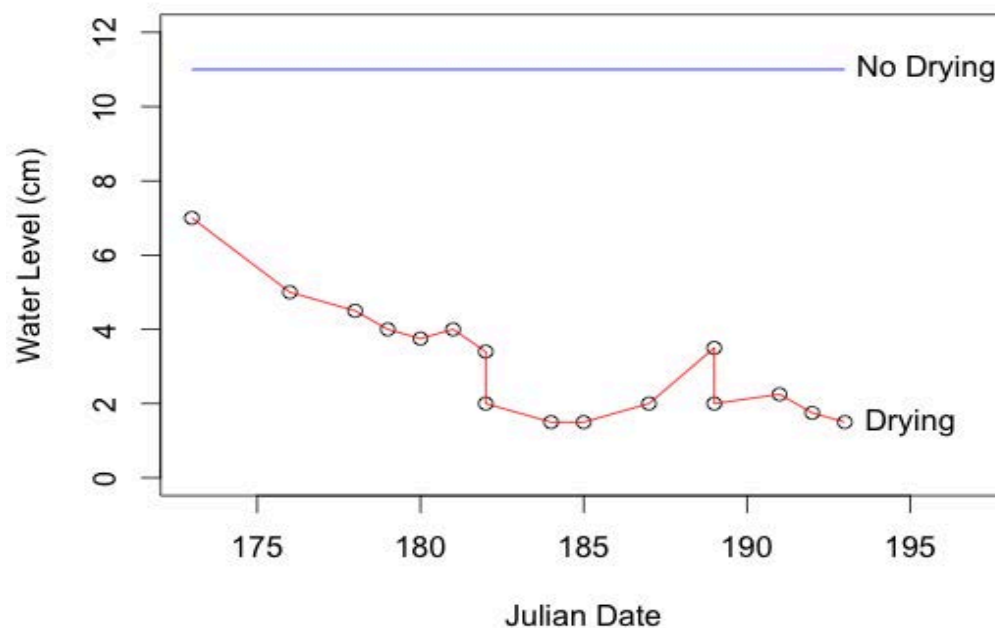


Figure 1: Water level of treatments over time of experiment

Behavior	Description
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Interaction	Total number of interactions
Aggression	Number of aggressive interactions including proleg wrestling, case shaking, and biting
% Aggression	Proportion of total interactions that are aggressive
Escalation	Number of aggressive interactions lasting more than 3 seconds
% Escalation	Proportion of aggressive interactions that escalated
Time Active	Amount of time spent crawling or interacting during observation
Mobbing	Number of cannibalistic mob events (with at least 5 larvae)
Cannibalism	Explicitly observing a larvae being eaten

Figure 1: Ethogram of measured caddisfly larvae behaviors



Image 1: Temporary pond shrinking in size at the Mexican Cut Nature Preserve

Observation	Dependent Variable	Independent Variable	F	df	p-value
Focal	Ln(Interaction)	Protein	4.219	1	0.04328
Focal	Aggression	Drying	13.236	1	0.000393
Focal	Aggression	Protein	5.191	1	0.0243812
Focal	% Aggression	Drying	7.2439	1	0.009705
Focal	% Aggression	Drying x Protein	4.0549	1	0.049551
Focal	Ln(Escalation)	Drying	4.0748	1	0.0469198

Scan	Aggression	Drying	8.8535	1	0.004609
Scan	Escalation	Drying	9.6881	1	0.003153

Table 2: Significant ANOVA results for experiment 1

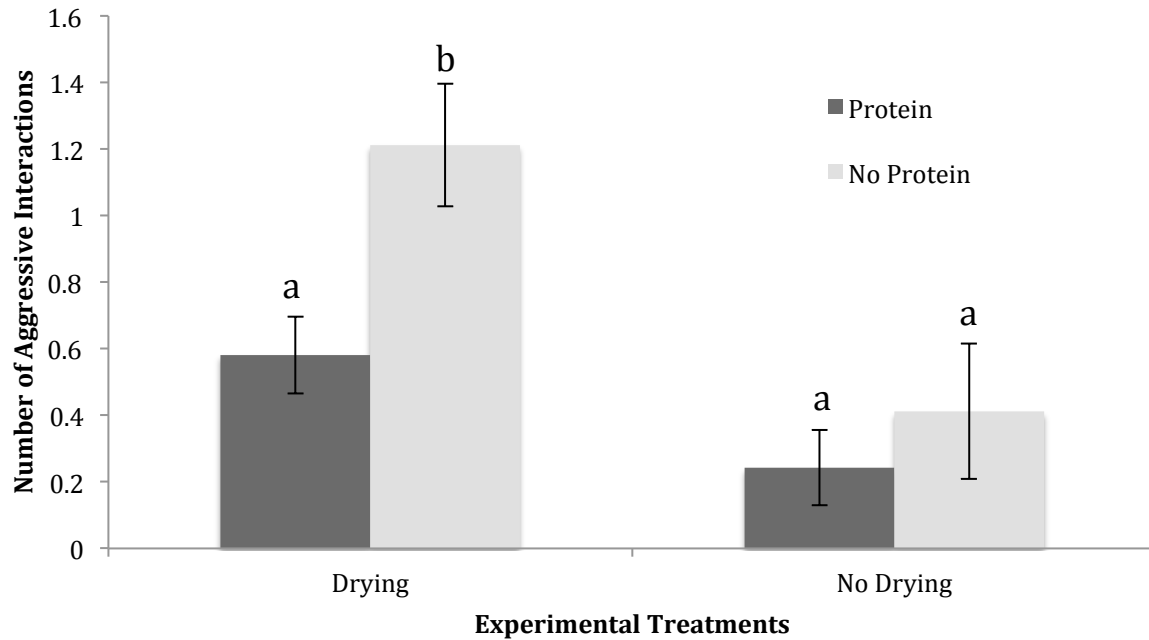


Figure 2: Aggressive encounters during 5' focal observations between drying and protein treatments. Two-sample ANOVA: significant effect of drying: $F=13.236$, $df=1$, $p\text{-value}=0.000393$; significant effect of protein: $F=5.191$, $df=1$, $p\text{-value}=0.0244$. Statistical differences between treatments were obtained with post-hoc pairwise t-tests.

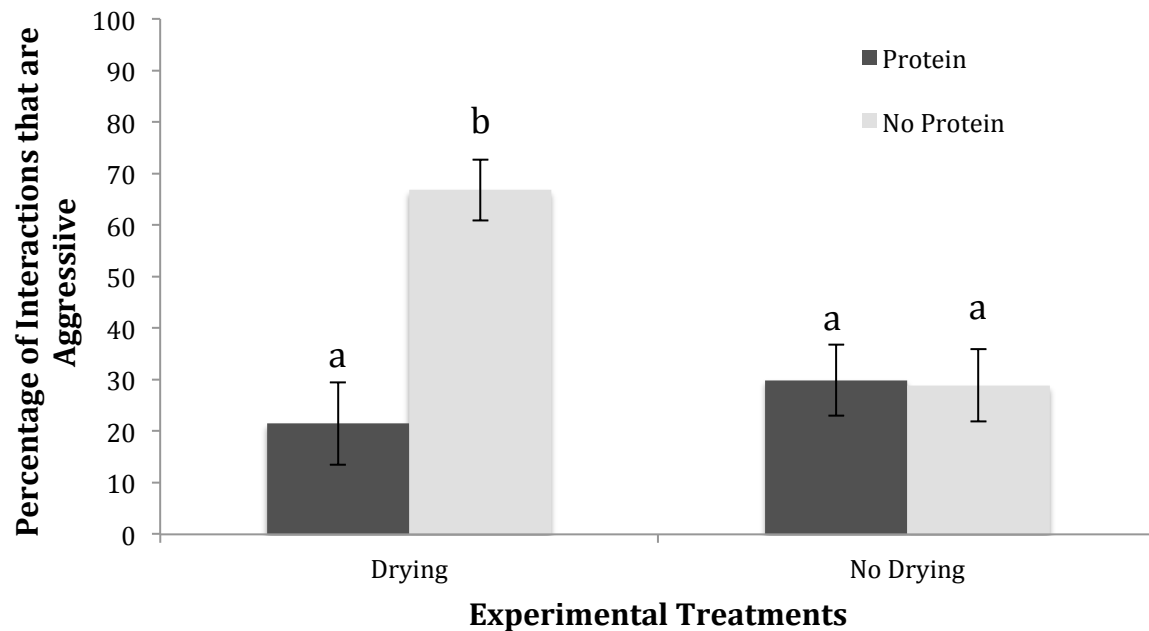


Figure 3: Percent aggressive interactions during 5' focal observation between drying and protein treatments. Two-sample ANOVA: significant effect of drying: $F=7.2439$, $df=1$, $p\text{-value}=0.009705$; significant interaction of drying $\times$

protein: $F=4.0549$, $df=1$, $p\text{-value}=0.049551$. Statistical differences between treatments were obtained with post-hoc pairwise t-tests.

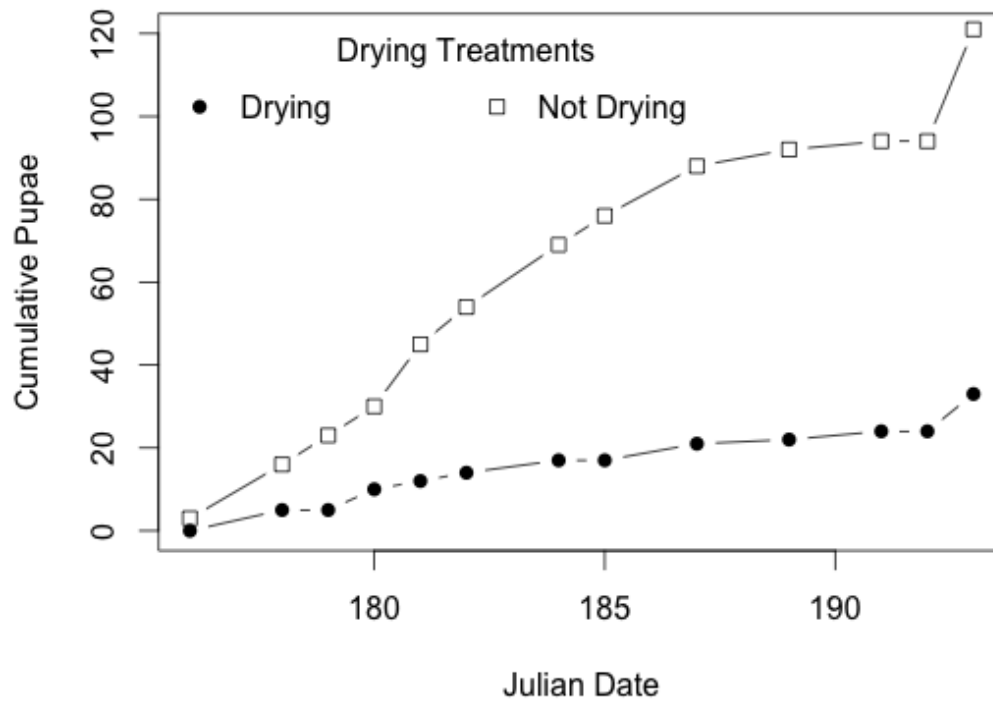


Figure 4: Number of pupae accumulated over time in drying and not drying treatments.

Observation	Dependent Variable	F	df	p-value
Focal	Escalation	13.12	3	0.000000777
Focal	Time Active	5.016	3	0.00338
Scan	Aggression	40.84	3	3.99E-15
Scan	Escalation	28.27	3	6.3E-12
Scan	% Escalation	3.682	3	0.0166
Scan	Mobbing	15.83	3	6.973E-08

Table 3: Significant ANOVA results for experiment 1

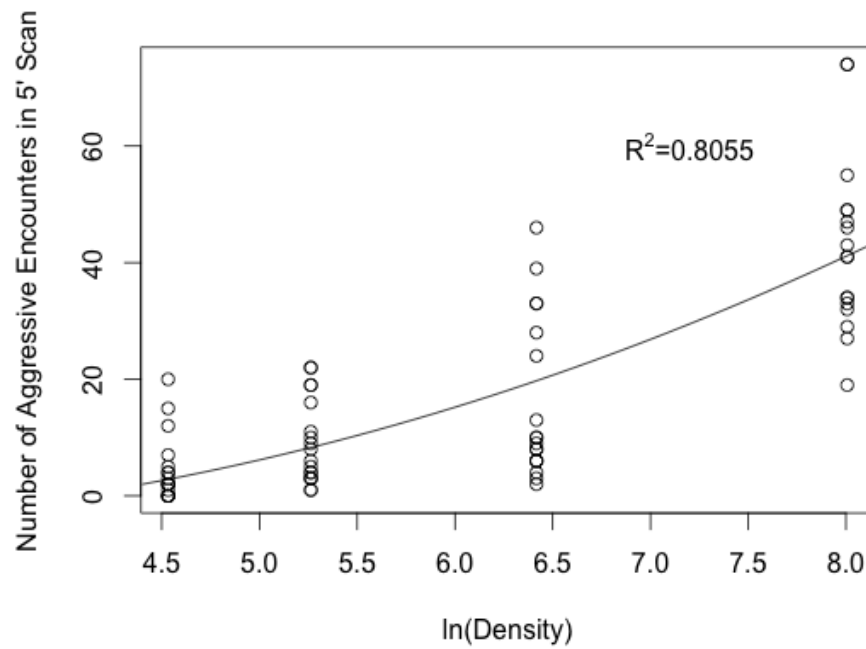


Figure 5: Number of aggressive interactions in scanning observation with natural log transformed density best fit to a 2nd order polynomial linear regression.

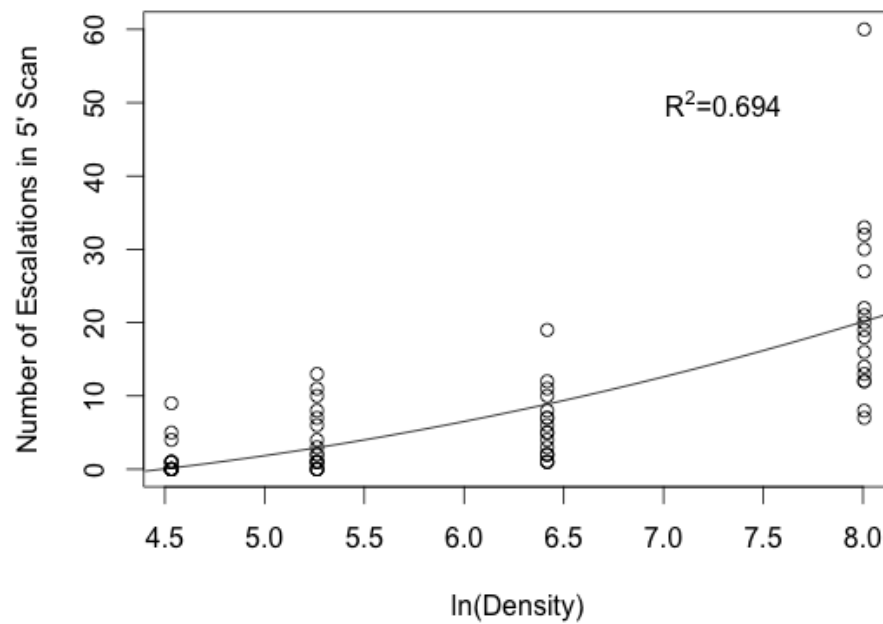


Figure 6: Number of escalations in scanning observation with natural log transformed density best fit to a 2nd order polynomial linear regression.

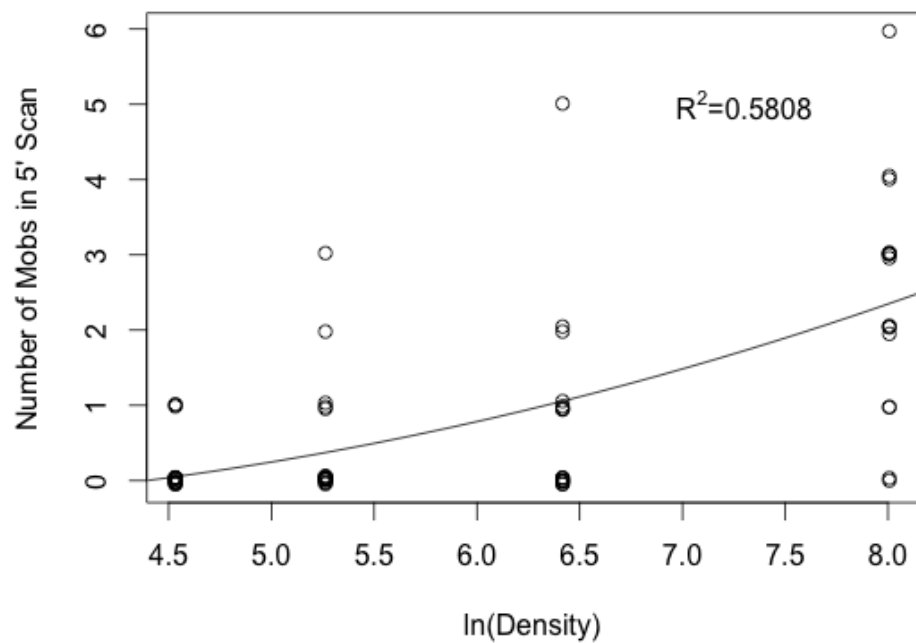


Figure 7: Number of mob events in scanning observation with natural log transformed density best fit to a 2nd order polynomial linear regression.

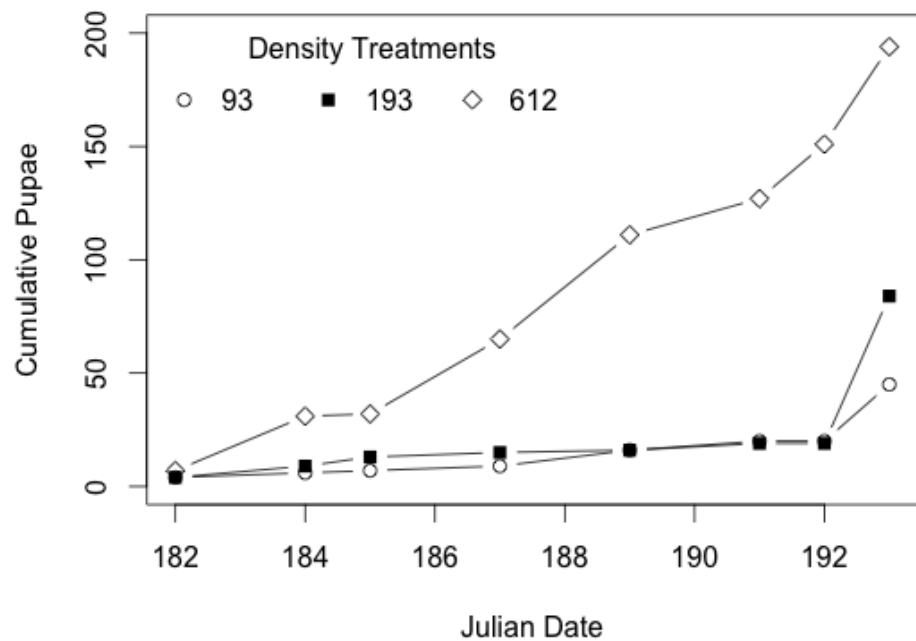


Figure 8: Number of pupae accumulated over time in density treatments