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# Effect of Fluctuating Temperatures on the Development of a Forensically Important Blow Fly, *Protophormia terraenovae* (Diptera: Calliphoridae)

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# Effect of Fluctuating Temperatures on the Development of a Forensically Important Blow Fly, *Protophormia terraenovae* (Diptera: Calliphoridae)

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**ABSTRACT** Experiments were conducted to compare the immature development of *Protophormia terraenovae* (Robineau-Desvoidy) at fluctuating temperatures of 4–28 and 9–23°C to their mean constant temperature, 16°C. Overall development was fastest at the greater fluctuation and slowest at the constant temperature but showed similar percentages of development time in each stage. The rate summation effect is suspected to have caused this difference in development rate because fluctuations above the mean increase the rate relatively more than temperatures below the mean can lower the rate.

**KEY WORDS** *Protophormia terraenovae*, fluctuating temperatures, rate summation effect, forensic entomology, Calliphoridae

Understanding time of death in a homicide is vitally important to allow investigators to investigate the timeline before death, as well as to verify or refute an alibi (Anderson 2001). A common method used to estimate elapsed time since death today involves the study of the development and changing community of carrion insects that colonize remains. Forensic entomology is the study of insects as they relate to legal events. A subset of forensic entomology, medico-legal or medico-criminal entomology, is the study of the insects associated with a body, primarily to estimate the length of tenure of insects on the body, and so indicate the minimum postmortem interval. When >72 h have elapsed since death, forensic entomology is considered the most accurate and often the only means available to estimate the postmortem interval (Kashyap and Pillai 1989, Anderson and VanLaerhoven 1996, Anderson 2001, Bourel et al. 2003).

Frequently, insect development is studied at constant temperatures; however, ambient temperatures do not remain constant and fluctuate over the 24-h day following thermoperiods. In the natural environment, temperatures do not fluctuate in a predictable manner, which makes it difficult to simulate for experimental purposes. To study the influence of fluctuating temperatures on insect development, one of five methods must be relied upon (Howe 1967). A first method involves transferring the insects from one temperature to another after a period of time has lapsed. The second method, similar to the first, is to transfer the insects to different temperatures according to a set pattern, and the third is to record the temperatures of the natural environment by using a data logger or thermograph. The fourth method, which by no means emulates the natural environment, is to set up conditions where the insects develop at temperatures based on a gradient as a result of insect mobility. The fifth and final method, used here, is to set an incubator to the desired conditions for development and cycle conditions every 24 h (Howe 1967).

Researchers have compared the development rates of the blow fly *Protophormia terraenovae* (Robineau-Desvoidy) (Diptera: Calliphoridae) at fluctuating outdoor ambient temperatures with the development rates at a set constant mean temperature of 20°C (Clarkson et al. 2004). Similarly, the development of *P. terraenovae* was compared among other species, at alternating temperatures of 10–20°C and 15–26°C with that at the respective mean temperatures (Davies and Ratcliffe 1994). These researchers found that development was significantly faster at alternating temperatures. The effects of a cold episode on the development of *Calliphora vomitoria* (L.) and *Calliphora vicina* (Robineau-Desvoidy) was examined to discover the influence this would have on estimating a post-mortem interval (Ames and Turner 2003). It was found that the accumulated degree hours (ADH) were not the same for cold treated insects as for control insects at 20°C and that errors were multiplied when applying constant temperature ADH to insects that were raised at fluctuating temperatures (Ames and Turner 2003). The successional arrival of a total of 53 taxa, including *P. terraenovae*, was documented at low fluctuating temperatures at high altitudes on rabbit carcasses; however, the carcasses were not visited daily and so data were not collected regularly for

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*P. terraenovae* (de Jong and Chadwick 1999). Other research has been conducted to investigate the effects of fluctuating temperatures on some species of blow fly (Dallwitz 1984; Byrd and Butler 1996, 1997, 1998), but until now, no study has taken into consideration daily fluctuations to below the minimum temperature threshold for *P. terraenovae.*

This study was completed to determine whether daily temperature fluctuations affect the speed of development of *P. terraenovae*, but more specifically to determine whether a fundamental drop in overnight temperature to below 10°C halts development. Earlier research has confirmed that *P. terraenovae* cannot complete development at a constant temperature of 10°C (Warren 2006). This research takes into consideration the effects of night time temperatures that are lower than 10°C, which is a common scenario in homicide cases from British Columbia. The specific objectives of this research were to compare immature development rates for *P. terraenovae* at fluctuating temperatures from 4 to 28°C and 9–23°C with the common mean temperature of 16°C and to compare development rates with 24 and 14°C fluctuations.

## Materials and Methods

Insect colonies were established from insects collected in the Lower Mainland of British Columbia. The insects were collected using two separate methods. Inverted cone traps were built from hollowed coffee canisters, wire mesh, and plastic bags. They were baited with pieces of beef liver (Byrd and Butler 1996, 1997, 1998; Anderson 2000) and put out in different locations to catch blow flies of differing lineages. Also, immature blow flies were gathered from animal carrion. Once the insects reached the adult stage, they were anesthetized or chilled to immobilize so that identification could be made under a dissecting microscope (EMZ-5TR; Meiji Techno, Santa Clara, CA). As the *P. terraenovae* were identified, they were placed into four different $(75\text{-cm})^3$ cages. Subsequent generations were used in the experiments.

The adult or stock colonies were maintained with provisions of a food source of 50:50 milk powder and cubed sugar as well as water ad libitum (Byrd and Butler 1996, 1997, 1998). As recommended, the water was only accessible by dental wicks to avoid drowning (Byrd and Castner 2001).

Beef liver, the rearing medium, was placed into black film canisters that were placed subsequently into each of the four stock cages, providing an ideal oviposition environment (Grassberger and Reiter 2001, 2002). Eggs were collected from the stock cage within 1.5 hr of the start of oviposition to ensure that development occurred in the desired environments (Howe 1967). The eggs were divided between control and experimental samples under a dissecting microscope. Samples were not held underneath the microscope for more than 10 min. A moistened children's paintbrush (size three, natural round; Liquitex, Cincinnati, OH) was used to transfer the eggs to each of the rearing containers.

**Table 1. The environmental chamber settings for the experiments set at fluctuating temperatures**

| Time | Incubator settings | | | |
|---|---|---|---|---|
| | 4 to 28°C | | 9 to 23°C | |
| | 12:12 (L:D), 75% RH | | 12:12 (L:D), 75% RH | |
| | Temperature | Lighting | Temperature | Lighting |
| 0:00 | 4°C | Off | 9°C | Off |
| 4:00 | 12°C | Off | 12.5°C | Off |
| 8:00 | 20°C | On | 19.5°C | On |
| 12:00 | 28°C | On | 23°C | On |
| 16:00 | 20°C | On | 19.5°C | On |
| 20:00 | 12°C | Off | 12.5°C | Off |
| 23:59 | 4°C | Off | 9°C | Off |

The eggs collected from the stock colonies were divided into batches of roughly 100 and placed into separate 4.5-liter wide-mouthed glass rearing containers each holding 125 ml of pine sawdust dampened with 15 ml of water. The purpose of the dampened sawdust was to allow for a migration medium and to prevent desiccation of the developing larvae. A paper towel was placed on top of the sawdust and under ≈200 g of beef liver to act as an interface between the sawdust and the beef liver. The paper towel prevented the eggs and larvae from drowning by soaking up any extra fluids that may have been released by the larval rearing medium. Approximately 100 eggs were placed on the surface of the beef liver in each of the rearing containers and the containers were kept secure using industrial paper towel lids and elastic bands. Larval aggregations can cause temperature elevation, but this was not an issue with so few larvae. Eggs from each cage were placed into jars such that a jar from each colony was added to each temperature chamber.

Environmental chambers (E7/2 plant chambers; Conviron, Winnipeg, MB, Canada) were used to maintain the thermoperiod (set over a 24-h clock), 75 ± 5% RH, and a photoperiod of 12:12 (L:D) h. The computerized environmental chambers maintained the required settings and a Smartbutton data logger (ACR Systems Inc., Surrey, BC, Canada) was used to record the temperature of the chamber at intervals of 30 min. In addition, a mercury thermometer (Fisherbrand, Houston, TX) was used to confirm the temperature settings of the chamber as it could easily be used to examine the temperature in the chamber without having to remove the data logger from the chamber.

Environmental chambers were set to maintain experimental temperature ranges from 4 to 28°C and 9–23°C with a control constant temperature of 16°C. The experimental temperatures were set at a range of 4–28°C and 9–23°C to imitate a rare drastic temperature change and one somewhat more likely, respectively. These are temperature fluctuations that we have witnessed in connection with actual cases. The experimental chamber temperatures were programmed to drop drastically during the "evening" section of the cycle (Table 1). The temperature settings for each incubator followed the same patterns during the entire experiment. At those settings, the 4–28°C chamber followed an actual 24-h pattern of 8 hr at 4°C;

**Table 2. The mean minimum developmental times for *P. terraenovae* to reach each stage at fluctuating temperatures of 4 to 28°C, 9 to 23°C, and their mean constant temperature 16°C**

| | Mean (days ± SE) | | | | | |
|---|---|---|---|---|---|---|
| | 4 to 28°C | ± SE | 9 to 23°C | ± SE | 16°C | ± SE |
| First instar | 1.75 | 0 | 1.75 | 0 | 2.57 | ±0.23 |
| Second instar | 3.75 | 0 | 5.06 | ±0.06 | 5.88 | ±0.31 |
| Third instar | 5.62 | ±0.12 | 8.75 | ±0.41 | 9.17 | ±0.58 |
| Post feeding | 9.06 | ±1.01 | 13.06 | ±0.59 | 15.08 | ±1.3 |
| Pupal | 12.38 | ±0.3 | 18.0 | ±0.85 | 20.25 | 0 |
| Adult | 23.62 | ±0.59 | 32.25 | ±0.96 | 38.31 | ±1.22 |

an hour each at 7, 13, 19, and 25°C; 8 hr at 28°C; and finally an hour each at 25, 19, 13, and 7°C. The 9–23°C chamber followed a continuous pattern of 8 hr at 9°C; an hour each at 11, 14, 18, and 21°C; 8 hr at 23°C; and finally an hour each at 21, 18, 14, and 11°C. Thus, between drastic changes in temperature, an average temperature of 16°C always was maintained. The control chamber was set and maintained at a constant temperature of 16°C but followed the same relative humidity and photoperiod, 12:12 (L:D) h, as the experimental chambers.

In the fluctuating temperature experiments, temperature and lighting changes were set to follow a sinusoidal curve and consequently, were similar to that of the natural environment. When temperature is plotted with a poikilothermic animal's development rate, it follows this same sinusoidal curve (Davidson 1944). The photoperiod was set to coincide with that of the thermoperiod, given that they naturally cycle together (Beck 1983). A sinusoidal setting for both the thermoperiod and photoperiod were followed so that the lights turned off as the colder temperatures were approached and turned on as the warmer temperatures were approached.

All trials (jars containing eggs) were added to the chambers at the same time, within 2 hr of the beginning of oviposition, so that exposure was the same for all insects, because blow flies develop at different rates at different stages of their life cycle (Levot et al. 1979, Williams 1984).

Every 12 h, close to the photoperiod change from light to dark or vice versa and sometimes more frequently, the insects were examined. Generalizations about the insects' appearances were made and the stage of development was established. Larval stages were determined based on the number of spiracular slits. Observations about the populations were made. As the insects reached each stage of development, times were recorded and general notes were made about the stage. Development was observed and noted from hatch to emergence as an adult fly at each of the temperatures.

The temperature of 16°C was chosen as the control because it falls within the linear section of the development rate versus temperature curve (Warren 2006) for *P. terraenovae* and it is a mean temperature that is regularly seen in cases during insect season in the Lower Mainland. To remove humidity as a variable, a relative humidity of 75% was selected as it is a known humidity used in fluctuating temperature experiments for *P. terraenovae* of the Lower Mainland of British Columbia (Clarkson et al. 2004).

As suggested by previous authors, sampling occurred every 12 h during each period shift of the photoperiod (Byrd and Butler 1996, 1997, 1998) with the exception of times when a molt was imminent. Occasionally, when a molt was expected, the insects were observed more frequently in an attempt to capture the change.

Recently laid eggs collected from each cage were divided into three separate jars so that ≈100 eggs were placed into each jar. One jar was placed into each of the experimental chambers and one into the control chamber. Subsequent generations of four separate *P. terraenovae* stock colonies were used such that there were four repetitions of the same experiment at the same time. The eggs that were oviposited in cage A became experiment A in each of the chambers, the eggs that were collected from cage B became experiment B in each chamber, and so forth, to ensure that there was no bias caused by stock colonies.

Development of *P. terraenovae* in each jar was observed. Both the minimum developmental times and mode of development were recorded, in days, for each stage. Comparisons of this development were made between the two fluctuating temperature regimes and the common mean temperature of 16°C.

## Results

The mean minimum development times and mode of development times to reach each of the development stages are presented in Tables 2 and 3. In all four jars maintained at temperatures fluctuating from 4 to 28°C, the minimum number of days to hatch was 1.75 and the minimum number of days to molt to second instar was 3.75 d. In fact, development was consistent among the jars up to and almost including third instar. A small difference was seen in jar 1 as the postfeeding stage was reached and this difference was maintained at the following stages.

The minimum developmental times were similar to the mode of development times at 4–28°C. However, the only real consistency among the jars for mode of development is to second instar at 3.75 d.

In every jar of insects raised at 9–23°C, the minimum developmental time to reach first instar was identical to those raised at 4–28°C; that is, 1.75 d. Jars 3 and 4 demonstrated similar development through the experiment and jar 1, containing the insects that

**Table 3. The mode of development for *P. terraenovae* to reach each stage at fluctuating temperatures of 4 to 28°C, 9 to 23°C, and their mean constant temperature 16°C**

| | Mean (days ± SE) | | | | | |
|---|---|---|---|---|---|---|
| | 4 to 28°C | ± SE | 9 to 23°C | ± SE | 16°C | ± SE |
| First instar | 2.25 | ±0.2 | 2.0 | ±0.25 | 3.19 | ±0.19 |
| Second instar | 3.75 | 0 | 5.25 | ±0.18 | 6.25 | ±0.29 |
| Third instar | 6.44 | ±0.24 | 9.12 | ±0.38 | 11.08 | ±0.67 |
| Postfeeding | 10.06 | ±0.8 | 15.62 | ±0.12 | 18.08 | ±0.73 |
| Pupal | 13.88 | ±0.43 | 19.38 | ±1.03 | 22.0 | ±0.31 |
| Adult | 25.56 | ±0.49 | 36.08 | ±1.34 | 40.0 | ±0.63 |

developed the slowest, and Jar 2, containing the insects that developed the fastest, were not that dissimilar.

In jars 1, 3, and 4, only 15, 17, and 15 adult flies emerged, respectively, at 4–28°C. Approximately five flies in each jar did not emerge from their puparia. However, in jar 2, 72 adult flies from ≈100 eggs emerged and 10 reached only as far as the pupal stage.

The mode of development at temperatures fluctuating from 9 to 23°C in jars 3 and 4 showed similar development with only slight variation to reach each of the stages. Jar 1, however, in general, developed slower than the other jars, and jar 2 developed faster at the later stages. Furthermore, in jar 2, *P. terraenovae* had two modes of development at the adult stage and both were earlier than the other jars (30.75 and 31.25 d).

In jars 3 and 4 only 46 adult flies emerged along with five adult flies with incomplete emergence, and 51 adult flies emerged along with 18 adult flies with incomplete emergence, respectively. In jar 1, 147 flies emerged and six remained in their pupal case. However, in jar 2, >200 flies emerged.

The temperature in the 16°C chamber was rather unstable at the beginning of the experiments and so the experiment in that particular chamber was stopped and restarted 4 d later when the temperature was stabilized. However, the adult flies in the stock colony cages laid their eggs for the new set of experiments only 1 hr later in the thermoperiod from the time of oviposition 4 d previously. The hour difference was thought to be negligible as the temperature did not change in the 16°C chamber, and the insects still were added during the dark fluctuation of the photoperiod as they had been when they were added to the fluctuating temperature chambers.

The minimum developmental time at 16°C in jar 3 differed radically from the development in the other jars and the insects took much longer to reach each stage. Unfortunately, the minimum developmental times to reach each of the third instar, postfeeding, and pupal stages were missed in this jar and, therefore, the mean development is based only on three jars. The third instar was missed because the insects were hidden among the folds of the liver and paper towel. The postfeeding and pupal stage both were missed because finding the insects in among the sawdust on the bottom of the jar proved to be difficult. At the time, it was decided that the results from three jars would be sufficient if the change in insect stage was missed and that it was better to keep the insects in the temperature chambers.

Although the mode of development to reach third instar and the pupal stage was missed in jar 3, the mode of development at 16°C was observed at the pupal stage (unlike the minimum development time) and was determined to be 22 ± 0.31 d. In jar 3, only five flies emerged at the end of the experiment and one fly only partially emerged from its pupal casing. In jar 1, 75 flies emerged and four flies did not develop beyond pupae. In jar 4, 142 flies emerged and 15 flies did not develop beyond pupae. Finally, in jar 2, 167 flies emerged and 10 flies did not develop beyond the pupal stage.

The tables show the developmental time differences observed at the fluctuating temperatures compared with each other and to their common mean temperature. They also illustrate that development was fastest at the fluctuating temperatures of 4–28°C each time, except for the mode of development to the first instar, which was earliest at fluctuations of 9–23°C.

Fig. 1 illustrates the minimum developmental time (a) and mode of development (b) for *P. terraenovae* in each stage of development expressed as a percentage of complete immature development for fluctuating temperatures of 4–28°C and 9–23°C along with their common mean temperature of 16°C. Interestingly, the percentage of time spent in each stage at 16°C is quite similar to the average time spent in each stage at each of the three temperature regimes. That is, unlike that derived at fluctuating temperatures, the percent of time spent in each stage at 16°C does not deviate greatly from the mean.

Clearly, the longest stage of development is the pupal stage. During complete immature development, the insects spend approximately half their time metamorphosing into an adult blow fly. The shortest stage at all three temperature regimes was the egg stage for both the minimum development time and for the mode of development. For the most part, the percentage of development in each stage is similar among the temperatures and only shows some variation at the mode of development. In the egg stage, the percent of immature development is much less at temperatures fluctuating from 9 to 23°C. It is only 5.9% as compared with 8.8 and 8% at temperatures of 4–28 and 16°C, respectively. At first instar, *P. terraenovae* spends only ≈6% of its development at 4–28°C as compared with 9.5 (9–23°C) and 7.8% (16°C). At the postfeeding stage, *P. terraenovae* spends a greater percentage of

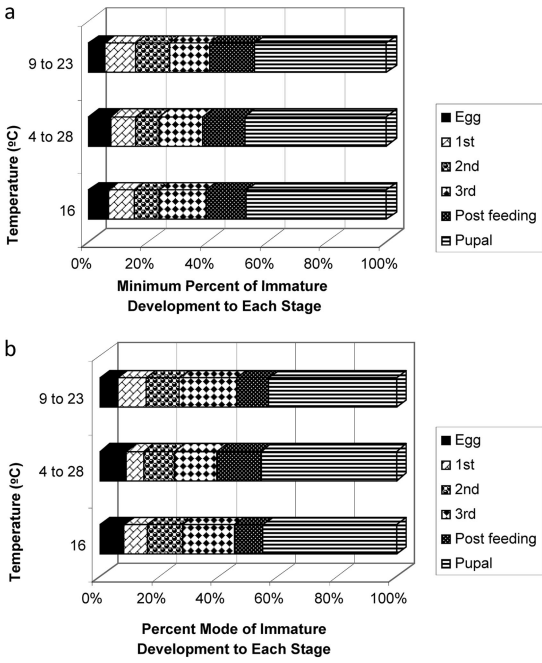


**Fig. 1.** The minimum developmental time (a) and mode of development (b) for *P. terraenovae* in each stage of development expressed as a percentage of complete immature development for fluctuating temperatures of 4–28 and 9–23°C and their mean constant temperature of 16°C.

time at temperatures fluctuating from 4 to 28°C than it does at the other two temperature regimes.

## Discussion

Early fluctuating temperature experiments began with rather simplistic devices (Hagstrum and Hagstrum 1970) or relied on the uncontrolled natural environment itself (Howe 1967) to provide temperature fluctuations, but now depend on devices such as the environment chambers used here. The chambers were set to follow the 24-h fluctuation patterns as set out in Table 1. The chamber set for fluctuations of 4–28°C was most accurate according to the recordings of the data logger and frequent testing by using a mercury thermometer. In the chambers set for 16 and 9–23°C, the temperatures infrequently varied from their settings. Minor fluctuations occurred at these temperatures, but this was deemed acceptable as ambient temperatures vary naturally (Liu et al. 1995). In the chamber set for fluctuations of 9–23°C, temperatures were recorded by the data logger as low as 6°C and fell once to as low as 5.5°C. However, because of frequent checks with the thermometer, this was not a regular occurrence and the experiment was continued.

The insects were added to each chamber at the same time and therefore at the same time in the scheduled fluctuating temperatures. It just so happened that when the eggs were laid it was during the coldest temperature of the thermoperiod, hence they were added at 4, 9, and 16°C. Addition to the chambers at the coldest temperature may affect egg development, and further research should be done to look at the differences that occur to development when the insects are added to the environmental chambers at a different time in the thermoperiod.

Fig. 1a and b indicate that the percent of development in each stage is similar at all three temperature settings. The greatest percent of immature development occurs at the pupal stage, at ≈50%. Also, the percent of development in each stage at the mean temperature, 16°C, is almost exactly the same as the mean percent of development for all three temperature regimes. Although some deviation from the mean percent occurs at 4–28 and 9–23°C, it does not occur at 16°C.

In some cases, large differences were seen between jars, in both developmental times and emergence rates. The latter may be explained by the fact that egg numbers were estimated rather than counted. In future studies, eggs should be lightly floated and separated on graph paper so that they may be observed under a dissecting microscope and divided into more equal samples (Wells and Greenberg 1992). Despite this, differences clearly were seen between experiments. This may have many causes, but may suggest that a greater variation in developmental times occurs between insects raised at greatly fluctuating temperatures compared with those raised at a constant temperature. It is probable that much larger replicates will be required to fully elucidate this.

The results found here indicate that *P. terraenovae* develops faster at fluctuating temperatures than it does at the mean constant temperature, which is similar to previous findings for *P. terraenovae* (Davies and Ratcliffe 1994). This, however, differs from other work that showed that development was faster to first-, second-, and third-instar stages at constant temperature and that there was no difference to pupal and adult stages (Clarkson et al. 2004). In fact at all stages, as indicated by Tables 2 and 3, development was fastest at extreme fluctuations of 4–28°C and slowest at 16°C. One exception was the minimum development time to first instar at 4–28°C, which was the same under both fluctuating temperature regimes. In addition, the mode of development for *P. terraenovae* to reach first instar at a fluctuation of 9–23°C ($2.0 \pm 0.25$) was less than the mode of development required at 4–28°C ($2.25 \pm 0.20$). Because *P. terraenovae* experiences a greater overall amplitude at fluctuations of 4–28°C than it does at 9–23°C, the insects will develop at a faster rate when the temperature is climbing from a lower temperature to a greater temperature, according to the rate summation effect. The rate summation effect is a phenomenon that is observed with sinusoidal development and fluctuating temperatures. Fluctuations at low temperatures to above and below a given mean temperature cause an increased development rate as compared with the mean temperature, because those fluctuations above the mean temperature increase the development rate relatively more than those fluctuations below the mean can lower the rate (Higley and Haskel 2001).

In conclusion, development was faster overall at the fluctuating temperatures and, therefore, further use of mean temperatures representing development at fluctuating temperatures must be done cautiously. Development occurred the fastest at the greatest temperature fluctuation (4–28°C) and was more comparable to the mean temperature development at a less drastic fluctuation. Perhaps the larger climb in temperature over the same length of time acted to boost development during each 24-h cycle. As a result of the greater amplitude of temperature exposure that the insects were subjected to in the 4–28°C chamber, and because of the rate summation effect, development may have come in spurts as temperatures rose from those lower temperatures below the mean temperature. The same would be true for the insects exposed to the 9–23°C fluctuations, but those insects only were exposed to temperatures over an amplitude of 14°C as compared with a change in temperature of 24°C. The insects in the 16°C chamber were exposed strictly to the mean temperature with only extremely minor fluctuations, and therefore were not exposed to any effects of sinusoidal fluctuating temperatures. Nevertheless, similar comparisons must be done at other temperatures to see if this explanation holds true. Research in the area of maggot masses and microclimates should be completed as the effect may not be as great, as the insects themselves may not be impacted by such extremes.

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