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The Influence of Photoperiod on Development Rates of Three Species of Forensically Important Blow Flies

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ABSTRACT. Environmental factors, such as temperature and photoperiod can play important roles in insect development. Numerous studies have researched insects and their responses to photoperiod, but there has been little research to show if the duration of light alters development rates in blow flies. A review of previous literature revealed differences in the light regime (L:D) reported for the study. This study examined the effects of photoperiod on development rates of *Phormia regina* Meigen, *Cochliomyia macellaria* F., and *Calliphora vicina* Robineau-Desvoidy using four light regimes of 0, 12, 16, and 24 h of light measured at both 20°C and 26°C. Environmental growth chambers were used to conduct the experiments with larvae reared on ground beef or beef liver inside 3.55-liter rearing containers. Using an analysis of variance, the results showed significant differences in development rates, from egg to adult emergence (d) among several treatments for each species at each temperature. There was a temperature by light interaction, and the data showed that the effects of light are magnified at lower temperatures. Development rates of all three species in the larval stage (egg to pupa) were the fastest when reared under cyclic light. Therefore, development rates determined from studies of forensically important species that used full light 24:0 (L:D) h may be too slow, i.e., the post mortem interval is inaccurate. The significance of this research will allow more accurate post mortem interval determinations in medico-legal criminal investigations by pinpointing the photoperiod where development rates occur the fastest.

Key Words: forensic entomology, photoperiod, PMI, Calliphoridae

Extrinsic factors such as ambient temperature, photoperiod, and other environmental factors play important roles in insect development. The cycles of activity and rest in animals are not due to a passive response but are driven by a preadapted internal clock. The endogenous clock that synchronizes the daily rhythms of animals to a period of 24 h is entrained by light, as well as temperature and insect species respond exogenously, where their activity is a response to the amount of light occurring regularly each day (Beck 1980, 1983). However, most insect activity rhythms have a physiological or endogenous basis that is partly independent of environmental signals (Daly et al. 1998).

Previous research has investigated insect responses to photoperiod, including Dipterans, but the literature does not clearly elucidate whether or not light alters development rates (Nabity et al. 2007). Some have focused research on how circadian rhythms affect insect behavior (Hong and Saunders 1994, Pyza and Cymborowski 2001) or influence of photoperiod on larval diapause (Vinogradova and Zinovjeva 1972; Nesin et al. 1995; McWatters and Saunders 1996, 1997; Vinogradova and Reznik 2002; Tachibana and Numata 2004). Photoperiod interacts with temperature, and most insects respond to the absolute length of the photoperiod, rather than the actual duration of the light period, where only short wavelengths are concerned in the photoperiodic reaction (Takeda and Skopik 1997, Chapman 1998). The effects of light in concert with temperature have shown variable duration and incidence of diapause in blow flies (Saunders 1997, McWatters and Saunders 1998, Saunders and Cymborowski 2003, Tachibana and Numata 2004, Muguruma et al. 2010). Photoperiodic sensitivity and diapause induction in *Calliphora vicina* Robineau-Desvoidy is largely maternal (Saunders et al. 1986, Vaz Nunes and Saunders 1989).

Flies in the family Calliphoridae will colonize a carcass within the first 2–3 h of exposure (Byrd and Castner 2001, Campobasso et al. 2001). Estimates of the post mortem interval (PMI) using entomological evidence, particularly blow fly biology, can be useful in criminal investigations. The majority of forensic entomology research thus far has focused on temperature, degree days, and insect succession patterns, but little research exists as to how photoperiod affects larval development rates. While observations have been noted, there were no direct evaluations of the effect of light on forensically important Calliphorid fly species (Nabity et al. 2007). However, recent work conducted on the effects of photoperiod on immature blow flies has shown that (L:D) does play a role in development. Mello et al. (2012) showed that while mean larval weight of *Chrysomya albiceps* (Wiedemann) did not vary between 24 h scotophase and 12 h photophase, larval, as well as pupal development time varied significantly between the two (L:D) cycles, and development was faster in the 24 h scotophase. Additionally, an increase in light regime from 12:12 (L:D) h to 24:0 (L:D) h led to prolongation of pupal time in both *Chrysomya megacephala* F. and *Chrysomya rufifacies* (Maquart) (Madhu and Devinder 2011). Muguruma et al. (2010) showed that photoperiod plays a role in expression levels of the two clock genes period (*per*) and timeless (*tim*) in *Protophormia terraenovae* Robineau-Desvoidy. The majority of previous research and temperature data has either failed to report the experimental photoperiod (Greenberg and Tantawi 1993; Anderson 2000; Grassberger and Reiter 2001, 2002a, 2002b; Grassberger and Frank 2003; Clarkson et al. 2004) or the data were generated under a 24:0 (L:D) h photo regime (Greenberg 1990, 1991; Byrd and Butler 1996, 1997, 1998; Byrd and Allen 2001; Huntington 2005; Nabity et al. 2006).

These previous studies still do not address the pertinent issues of how photoperiod and circadian rhythms affect development rates among the forensic indicator species of blow flies. The objective of this research was to conduct a more in-depth and comprehensive investigation to determine the effects of photoperiod on the development rates of three species of Calliphoridae. For this study, we hypothesized that the duration of light would play a distinct role in larval development.

Materials and Methods

For these experiments, three species of blow flies: *Phormia regina* (Meigen), *Cochliomyia macellaria* F., and *C. vicina* were used. *P. regina* larvae were collected from a calf carcass 3.2 km north of Montour, IA in Tama County (42°01' lat, 92°43' long) 12-VI-06 and reared to adults on beef liver. *C. macellaria* larvae were collected from pig carcasses near Rensselaer, IN, in Jasper County (40°56' lat, 87°00' long) 14-VII-06 and reared to adults on ground beef. *C. vicina* adults were collected in Lincoln, NE from the University of Nebraska-Lincoln East Campus in Lancaster County, NE (40°85' lat, 96°75' long) 25-X-06 using beef liver and aerial nets. Approximately 150–200 adults were kept in wire mesh cages (42 by 42 by 42 cm) in the environmental growth chambers (Models E-30B; I-35L, LLVL, VLX, Percival Scientific Inc., Perry, IA) to prevent overcrowding. *P. regina* and *C. macellaria* were kept at 25–26°C under 16:8 (L:D) h, while *C. vicina* were kept at 20°C and 12:12 (L:D) h, each with a relative humidity between 50 and 80%. Continual colonies survived on water and powdered sugar. Adults were provided beef liver for oviposition. Beef liver or ground beef (93% lean) was provided to colonies containing newly emerged adults for 3–4 d to facilitate ovary maturation and vitellogenesis. The chambers' fluorescent lights served as the light source.

Experiments were conducted from September 2006 to August 2007 using four different light regimes (16:8, 12:12, 24:0, and 0:24 [L:D] h), each at one of two temperatures, 20°C and 26°C. The experimental design was a factorial arrangement. The experimental unit was the environmental growth chamber. Treatments were assigned using a completely randomized design of light and temperature to chambers for each experiment. The species was selected randomly for each experiment, and each experiment was replicated four times over the course of several generations using four individual growth chambers. Rearing containers were used for immature development. The containers were 3.55-liter Rubbermaid (Atlanta, GA) clear tubs with lids (31 by 20 by 20 cm). Newly emerged adults were used to restock the colonies. In all experiments, fly development rates from egg to adult emergence were examined.

Approximately 100 eggs were used to reduce the effects of the maggot mass temperature influencing development rate. Eggs were collected within 3 h after oviposition, homogenized, and placed in aluminum boats inside four separate, individual 3.55-liter rearing containers. Each rearing container was randomly placed in a respective growth chamber, and the lids were vented to allow for gas exchange. Ground beef (93% lean) was provided as needed throughout larval development. The ground beef was allowed to attain the respective chamber temperature prior to placing in each container. Upon nearing pupation, vermiculite was added to the rearing container to facilitate dispersal and ultimately pupation. Temperatures of each rearing container were monitored using thermocouples (TMC6-HB, with 0–44°C range, ± 0.4°C accuracy at 20°C, and 0.2°C resolution) from Hobo H8 Outdoor/Industrial 4-Channel External Logger (Onset Computer Corp., Pocasset, MA). Rearing containers and larval stage were checked and recorded daily. Pupal and adult stage transition times using first emergence (60–70% of population emerged) were used. Larvae that were reared in 0:24 (L:D) h treatments were checked after sunset using a flashlight with a red light filter of ~650 nm.

Methods for recording temperature and calculating centigrade degree-days (CDD) were adopted from Nabity et al. (2006). The Hobo thermocouples recorded overall rearing container temperature at intervals of 15 min to the nearest 0.1°C. The 96 data points were averaged to obtain a mean daily temperature. A development threshold of 10°C was used to calculate CDD for *P. regina* and *C. macellaria*. A development threshold of 6°C was used to calculate CDD for *C. vicina*. The development thresholds were adopted from methods used by Kamal (1958) and Greenberg (1991). The sum of the CDD was used to obtain the accumulated degree-days (ADD) for the biological period (egg to adult) in each rearing container.

Data Analysis. Linear portions of the development curve, slope, and 95% confidence intervals were determined using linear regression analysis (GraphPad Prism 5, GraphPad Software, Inc.). Rearing container temperatures versus set chamber temperatures were analyzed using data from the thermocouples. Development times were compared and analyzed for significant differences using an analysis of variance at a significance level of $P \leq 0.05$ (PROC MIXED, SAS Institute 2003, Cary, NC).

Results

C. macellaria, *P. regina*, and *C. vicina* all developed faster at 26°C than 20°C (as expected) and development rates (d) differed when reared under each photoperiod, as indicated by differences among bar heights between 20°C and 26°C (Fig. 1), which represent the set growth chamber temperatures.

For *C. macellaria*, individual experimental unit (=chamber) values and means are presented for all treatment combinations of egg to pupa, pupa to adult, and egg to adult development in Table 1. Development of *C. macellaria* from egg to adult showed significant main effects of temperature ($F_{1, 21} = 569.14$, $P < 0.0001$), light ($F_{3, 21} = 8.30$, $P = 0.0008$), and the temperature by light interaction ($F_{3, 21} = 20.58$, $P < 0.0001$). Development from egg to pupa also showed significant main effects of temperature ($F_{1, 21} = 277.40$, $P < 0.0001$), light ($F_{3, 21} = 16.48$, $P < 0.0001$), and the temperature by light interaction ($F_{3, 21} = 16.23$, $P < 0.0001$). Finally, development from pupa to adult showed significant main effects of temperature ($F_{1, 21} = 135.25$, $P < 0.0001$). However, development from pupa to adult did not show significant differences by light ($F_{3, 21} = 1.37$, $P = 0.2799$) and by light and temperature interaction ($F_{3, 21} = 2.66$, $P = 0.0749$). Graphically, these results are illustrated in Figure 2, with associated equations from the linear regressions. Because the all dark treatment is not as biologically realistic as other treatments and may distort the regression, points, and regressions without the dark 0:24 (L:D) h treatment also are shown (Fig. 2). The slope of the linear regression in all treatments was significantly different from 0 at 20°C ($F_{1, 14} = 53.86$, $P < 0.0001$) but not at 26°C ($F_{1, 14} = 2.61$, $P = 0.1283$). Excluding the all dark treatment, the slope of the linear regression was significantly different from 0 at 20°C ($F_{1, 10} = 7.87$, $P = 0.0186$) but not at 26°C ($F_{1, 10} = 0.16$, $P = 0.6996$).

For *P. regina*, individual experimental unit (=chamber) values and means are presented for all treatment combinations in Table 2. Development of *P. regina* from egg to adult showed significant main effects of temperature ($F_{1, 21} = 635.66$, $P < 0.0001$), light ($F_{3, 21} = 84.30$, $P < 0.0001$), and the temperature by light interaction ($F_{3, 21} = 11.99$, $P < 0.0001$). Development from egg to pupa also showed significant main effects of temperature ($F_{1, 21} = 134.15$, $P < 0.0001$), light ($F_{3, 21} = 59.07$, $P < 0.0001$), and the temperature by light interaction ($F_{3, 21} = 10.67$, $P = 0.0002$). Development from pupa to adult showed significant main effects of temperature ($F_{1, 21} = 259.20$, $P < 0.0001$), light ($F_{3, 21} = 8.33$, $P = 0.0008$), and temperature by light interaction ($F_{3, 21} = 13.73$, $P < 0.0001$). Again, the all dark treatment is not as biologically realistic as the other light treatments and may distort the regression, as shown in Figure 3. The slope of the linear regression in all treatments did not show significant differences from 0 at 20°C ($F_{1, 14} = 3.61$, $P = 0.0783$) and at 26°C ($F_{1, 14} = 0.07$, $P = 0.7941$). Excluding the all dark treatment, the slope of the linear regression was significantly different from 0 at 20°C ($F_{1, 10} = 98.48$, $P < 0.0001$) and at 26°C ($F_{1, 10} = 9.25$, $P = 0.0125$).

For *C. vicina*, individual experimental unit (=chamber) values and means are presented for all treatment combinations in Table 3.
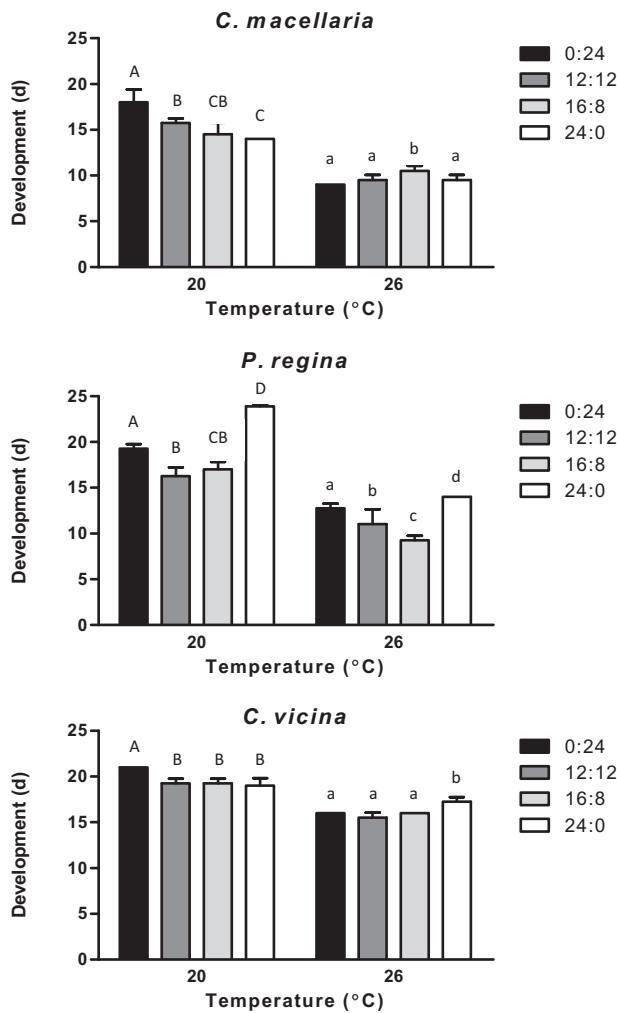
Fig. 1. Differences in development times (d) for *C. macellaria*, *P. regina*, and *C. vicina* from egg to adult under four light regimes using the set growth chamber temperatures of 20°C and 26°C. Means followed by the same letter above are not significantly different, and means with capital letters above are significantly different from means with lowercase letters between temperature regime (α = 0.05).

Development of *C. vicina* from egg to adult showed significant main effects of temperature ($F_{1, 21} = 367.20$, $P < 0.0001$), light ($F_{3, 21} = 6.27$, $P = 0.0033$), and the temperature by light interaction ($F_{3, 21} = 16.95$, $P < 0.0001$). Development from egg to pupa did not show significant main effects of temperature ($F_{1, 21} = 2.45$, $P = 0.1321$) but showed significant differences with light ($F_{3, 21} = 10.45$, $P = 0.0002$) and the temperature by light interaction ($F_{3, 21} = 18.45$, $P < 0.0001$). Development from pupa to adult showed significant main effects of temperature ($F_{1, 21} = 218.40$, $P < 0.0001$), light ($F_{3, 21} = 3.77$, $P < 0.0261$), and temperature by light interaction ($F_{3, 21} = 9.05$, $P = 0.0005$). Figure 4 shows the linear regressions for *C. vicina* with all treatments and without the all dark treatment since the all dark 0:24 (L:D) h treatment may distort the regression. The slope of the linear regression in all treatments was significantly different from 0 at 20°C ($F_{1, 14} = 30.87$, $P < 0.0001$) and at 26°C ($F_{1, 14} = 6.58$, $P = 0.0225$). The slope of the linear regression excluding the all dark treatment was not significantly different from 0 at 20°C ($F_{1, 10} = 2.59$, $P = 0.1385$) but was significantly different at 26°C ($F_{1, 10} = 36.52$, $P = 0.0001$).

Table 4 lists the ADD for each treatment from egg to adult. When comparing cyclic light to noncyclic, *C. macellaria* showed the

Table 1. Development time (d) for *C. macellaria* reared at set chamber temperatures of 20°C and 26°C, under four different light:dark (L:D) treatments

Chamber	Avg temp (°C)	(L:D) h	Egg to pupa	Pupa to adult	Egg to adult
1	26.71	0:24	5	4	9
2	26.85	0:24	6	3	9
3	26.65	0:24	6	3	9
4	26.75	0:24	6	3	9
Mean	26.74		5.8	3.3	9.0
SD	0.1		1.3	0.5	0.0
1	26.87	12:12	6	4	10
2	26.69	12:12	6	4	10
3	26.38	12:12	5	4	9
4	26.49	12:12	5	4	9
Mean	26.61		5.5	4.0	9.5
SD	0.22		0.58	0.00	0.58
1	26.36	16:8	6	5	11
2	26.29	16:8	6	5	11
3	26.64	16:8	6	4	10
4	26.73	16:8	6	4	10
Mean	26.51		6.0	4.5	10.5
SD	0.21		0.00	0.58	0.58
1	26.57	24:0	6	4	10
2	26.75	24:0	6	4	10
3	26.30	24:0	5	4	9
4	26.12	24:0	5	4	9
Mean	26.44		5.5	4.0	9.5
SD	0.28		0.58	0.00	0.58
1	20.51	0:24	13	7	20
2	20.81	0:24	10	7	17
3	20.00[a]	0:24	12	6	18
4	20.00[a]	0:24	11	7	18
Mean	20.66		11.5	6.8	18.3
SD	0.40		1.29	0.50	1.26
1	20.49	12:12	9	7	16
2	20.79	12:12	9	6	15
3	20.27	12:12	9	7	16
4	20.31	12:12	9	7	16
Mean	20.47		9.0	6.8	15.8
SD	0.24		0.00	0.50	0.50
1	20.49	16:8	8	5	13
2	20.39	16:8	8	6	14
3	20.42	16:8	8	8	16
4	20.58	16:8	8	7	15
Mean	20.47		8.0	6.5	14.5
SD	0.08		0.00	1.29	1.29
1	20.84	24:0	8	6	14
2	21.03	24:0	8	6	14
3	21.07	24:0	8	6	14
4	21.20	24:0	8	6	14
Mean	21.04		8.0	6.0	14.0
SD	0.15		0.00	0.00	0.00

Actual temperatures in larval rearing containers are reported by chamber (see Materials and Methods for details).
[a]Thermocouple malfunction. Set growth chamber temperature used.

largest difference among the 20°C treatments ($ADD_{0:24} = 207.64$, $ADD_{16:8} = 162.30$, $\Delta = 45.34$) and ($ADD_{0:24} = 207.64$, $ADD_{12:12} = 175.18$, $\Delta = 32.46$), but *C. vicina* showed a large difference as well ($ADD_{0:24} = 315.01$, $ADD_{16:8} = 297.46$, $\Delta = 17.55$) and ($ADD_{0:24} = 315.01$, $ADD_{12:12} = 291.28$, $\Delta = 23.73$), respectively. *P. regina*, for the 20°C treatments, showed the least difference ($ADD_{0:24} = 196.44$, $ADD_{16:8} = 181.70$, $\Delta = 14.74$) and ($ADD_{0:24} = 196.44$, $ADD_{12:12} = 177.52$, $\Delta = 18.92$). Additionally, differences in ADD were also observed for each species in the 26°C treatments when comparing cyclic to noncyclic light. *P. regina* showed the largest differences ($ADD_{0:24} = 233.41$, $ADD_{16:8} = 181.70$, $\Delta = 51.71$), ($ADD_{24:0} = 252.42$, $ADD_{16:8} = 181.70$, $\Delta = 70.72$), and ($ADD_{24:0} = 252.42$, $ADD_{12:12} = 204.79$, $\Delta = 47.63$). *C. macellaria* showed the least differences among the three species ($ADD_{0:24} = 167.40$, $ADD_{16:8} = 185.67$, $\Delta = 18.27$), ($ADD_{24:0} = 172.68$, $ADD_{16:8} = 185.67$, $\Delta = 12.99$), and
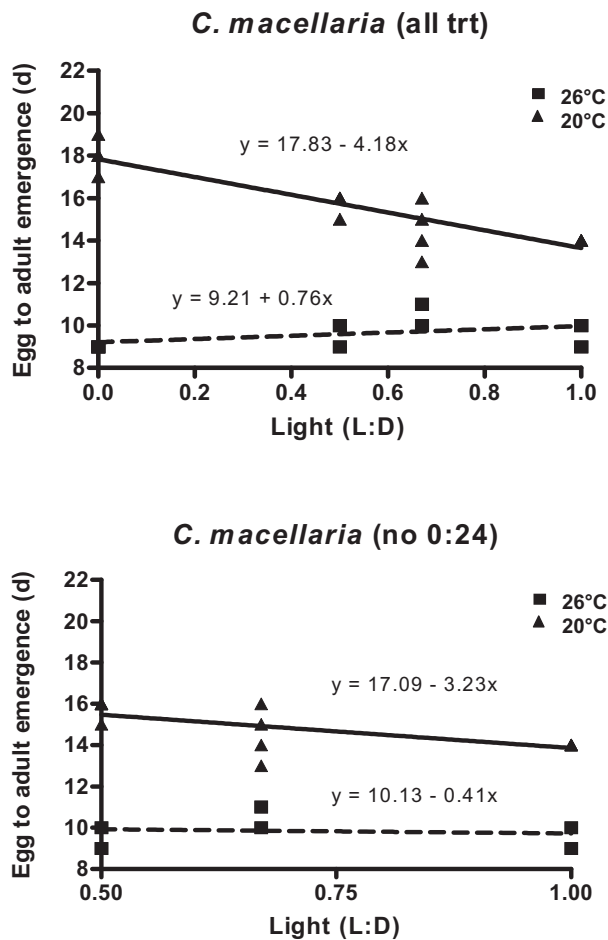
C. macellaria (all trt)

y = 17.83 - 4.18x

y = 9.21 + 0.76x

■ 26°C
▲ 20°C

C. macellaria (no 0:24)

y = 17.09 - 3.23x

y = 10.13 - 0.41x

■ 26°C
▲ 20°C

Fig. 2. Linear regression of *C. macellaria* development (d) from egg to adult using rearing container temperatures showing regression slopes for all treatments (all trt) and without the 0:24 (L:D) h treatment. $R^2 = 0.7937$ (20°C); $R^2 = 0.1573$ (26°C).

$(\text{ADD}_{24:0} = 172.68, \text{ADD}_{12:12} = 174.47, \Delta = 1.79)$. Differences for *C. vicina* were also observed $(\text{ADD}_{0:24} = 351.03, \text{ADD}_{16:8} = 346.47, \Delta = 4.56)$, $(\text{ADD}_{24:0} = 375.68, \text{ADD}_{16:8} = 346.47, \Delta = 29.21)$, and $(\text{ADD}_{24:0} = 375.68, \text{ADD}_{12:12} = 339.52, \Delta = 36.16)$. ADD for egg to pupa and pupa to adult was not calculated.

Discussion

Light influenced development rates for all species tested but not in the same way. Also in this study, there was a temperature by light interaction, and the effect of light was magnified at lower temperatures. As seen with a previous study, cyclic light caused larval development time to decrease with decreasing temperature for *P. regina* (Nabity et al. 2007). Lower temperatures are acting in concert with light to alter physiological responses in *C. macellaria* as well. For *C. macellaria* and *P. regina*, two warm-weather species, flies developed faster from egg to adult under cyclic light at both 26°C and 20°C. However, *C. vicina*, a cool-weather species, developed faster from egg to adult under cyclic light at 26°C only.

The two L:D treatments 0:24 h and 24:0 h represent extremes. The continuous dark or 100% absence of light represents a greater extreme than 100% light. The two extremes disrupt the normal circadian cycles, which would thus disrupt the flies' ability to cue in on either the photophase or scotophase, using light as a physiological trigger for stage transitions or as an emergence cue. Nabity et al. (2007) discussed the issue of emergence gating and concluded that development of *P. regina* did not correlate to photophase since no pattern of stage transition times occurring in groups was evident and that the increase in development

Table 2. Development time (d) for *P. regina* reared at set chamber temperatures of 20°C and 26°C, under four different light:dark (L:D) treatments

Chamber	Avg temp (°C)	(L:D) h	Egg to pupa	Pupa to adult	Egg to adult
1	26.88	0:24	8	5	13
2	26.90	0:24	8	5	13
3	27.02	0:24	8	5	13
4	27.11	0:24	10	2	12
Mean	26.98		8.5	4.3	12.8
SD	0.11		1.00	1.50	0.50
1	27.12	12:12	8	3	11
2	26.98	12:12	8	3	11
3	27.18	12:12	6	3	9
4	27.01	12:12	6	5	13
Mean	27.07		7.5	3.5	11.0
SD	0.09		1.15	1.00	1.63
1	26.00[a]	16:8	5	4	9
2	26.00[a]	16:8	5	4	9
3	26.00[a]	16:8	6	3	9
4	26.94	16:8	7	3	10
Mean	26.83		5.8	3.5	9.3
SD	0.47		0.96	0.58	0.50
1	26.78	24:0	11	3	14
2	26.83	24:0	11	3	14
3	26.83	24:0	11	3	14
4	26.88	24:0	11	3	14
Mean	26.83		11.0	3.0	14.0
SD	0.04		0.00	0.00	0.00
1	19.48	0:24	11	8	19
2	19.56	0:24	11	8	19
3	19.86	0:24	12	8	20
4	19.90	0:24	11	8	19
Mean	19.70		11.3	8.0	19.3
SD	0.21		0.50	0.00	0.50
1	20.36	12:12	9	7	16
2	20.47	12:12	8	7	15
3	20.20	12:12	9	8	17
4	20.16	12:12	9	8	17
Mean	20.30		8.8	7.5	16.3
SD	0.14		0.50	0.58	0.96
1	19.97	16:8	11	7	18
2	19.99	16:8	11	6	17
3	20.07	16:8	11	6	17
4	20.38	16:8	10	6	16
Mean	20.10		10.8	6.3	17.0
SD	0.19		0.50	0.50	0.82
1	20.55	24:0	13	10	23
2	20.53	24:0	13	11	24
3	20.49	24:0	13	11	24
4	20.49	24:0	14	10	24
Mean	20.52		13.3	10.5	23.8
SD	0.03		0.50	0.58	0.50

Actual temperatures in larval rearing containers are reported by chamber (see Materials and Methods for details).
[a]Thermocouple malfunction. Set growth chamber temperature used.

time under cyclic light was more important than the issue of gating. It is plausible to suggest that, like plants, calliphorids may only require a small amount or short duration of light to facilitate development and stage transitions. Achieving a 100% absence of light in an experimental setting is difficult, and it is possible that merely opening the environmental chamber door to check the larvae, despite best efforts to eliminate all artificial light, provides enough light for the flies to reset their circadian clocks.

Development rates of *C. macellaria* from egg to pupa were fastest not only under cyclic light at both 26°C and 20°C but also at constant light 24:0 (L:D) h at both 26°C and 20°C (Table 1). The larvae developed to pupae in 5.5 d (SE 0.2919) at 26°C under both 12:12 (L:D) h and 24: 0 (L:D) h, and 8.0 d (SE 0.2919) at 20°C under 16:8 (L:D) h and 24:0 (L:D) h. However, adults emerged fastest under the 0:24 (L:D) h in 9.0 d (SE 0.3520) at 26°C but fastest under the 24:0 (L:D) h in 14.0 d (SE 0.3520) at 20°C. There was no significant difference $(t_{1, 21} = 1.00, P = 0.3266)$ between the 16:8 (L:D) h and 24:0 (L:D) h

P. regina (all trt)

$y = 17.03 - 4.4x$

$y = 11.54 - 30.11x$

- ■ 26°C
- ▲ 20°C

P. regina (no 0:24)

$y = 7.31 - 0.45x$

$y = 6.25 - 0.88x$
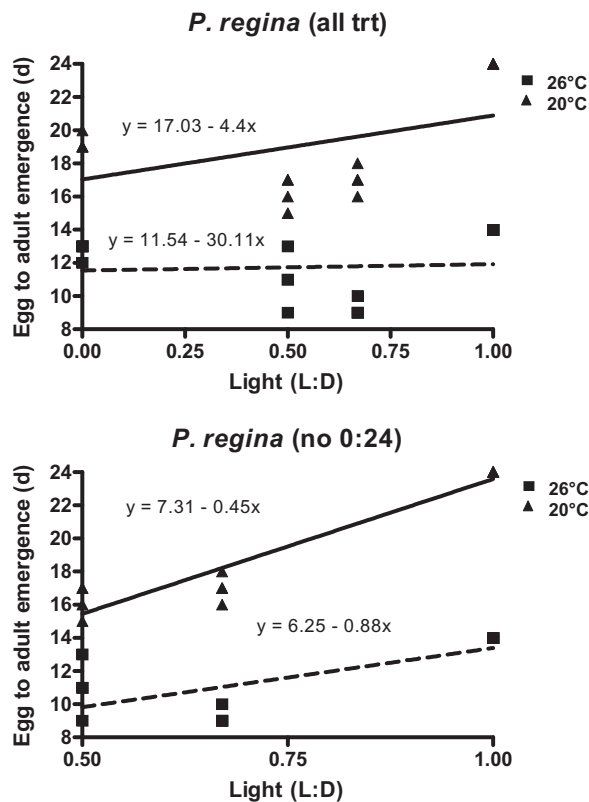
- ■ 26°C
- ▲ 20°C

Fig. 3. Linear regression of *P. regina* development (d) from egg to adult using rearing container temperatures showing regression slopes for all treatments (all trt) and without the 0:24 (L:D) h treatment. $R^2 = 0.2049$ (20°C); $R^2 = 0.0050$ (26°C).

treatments at 20°C. *C. macellaria* was significantly affected by light and the temperature by light interaction among the treatments at 20°C for both egg to adult and egg to pupa development, but only differences with regard to temperature were seen in development times from pupa to adult. The varying light regimes did not affect the development rates of *C. macellaria* at 26°C (Fig. 2). Figure 5 illustrates the linear regressions for development (d) from egg to pupa and pupa to adult. Since no significant differences were seen among the pupa to adult treatments in the 20°C with respect to light or the temperature by light interaction, this suggests that *C. macellaria* is responding to the effects of light in the larval stages. Specifically, light is affecting larval dispersal. The data for *C. macellaria* also support the conclusion that the effects of light are magnified at lower temperatures, citing the differences seen from the linear regression slopes between 26°C and 20°C treatments in Figure 2.

Development times from egg to adult, as well as from egg to pupation, were fastest under cyclic light (12:12 [L:D] h) for *P. regina* at both 26°C and 20°C. At 20°C, *P. regina* showed significant effects of temperature, light, and the temperature by light interaction for development times from egg to adult, egg to pupa, and pupa to adult. Under continuous light (24:0 [L:D] h) and at approximately 26°C, *P. regina* spent an average of 3 d (SE 0.3593) in the pupal stage and an average of 10.5 d (SE 0.3593) when reared at approximately 20°C. Yet, under cyclic light (16:8 [L:D] h), *P. regina* spent an average of 3.5 d (SE 0.3593) in the pupal stage at 26°C and an average of 6.3 d (SE 0.3593) at approximately 20°C. Comparing the results from the 16:8 (L:D) h and 24:0 (L:D) h treatments at 26°C, we see there were no significant differences ($t_{1, 21} = 0.441$, $P = 1.13$). These data support the conclusion that the effect of light on development rates is magnified at lower temperatures, as also predicted by Metzger and Rust (1996). Previous photoperiod research with *P. regina* showed that larval development was more affected by light than pupal development (Nabity et al. 2007).

Table 3. Development time (d) for *C. vicina* reared at set chamber temperatures of 20°C and 26°C, under four different light:dark (L:D) treatments

Chamber	Avg temp (°C)	(L:D) h	Egg to pupa	Pupa to adult	Egg to adult
1	26.73	0:24	8	8	16
2	26.71	0:24	8	8	16
3	26.67	0:24	8	8	16
4	26.59	0:24	8	8	16
Mean	26.68		8.0	8.0	16.0
SD	0.06		0.00	0.00	0.00
1	26.60	12:12	8	7	15
2	26.62	12:12	8	8	16
3	26.50	12:12	7	9	16
4	26.60	12:12	7	8	15
Mean	26.58		7.5	8.0	15.5
SD	0.05		0.58	0.82	0.58
1	26.39	16:8	7	9	16
2	26.30	16:8	7	9	16
3	26.45	16:8	7	9	16
4	26.38	16:8	7	9	16
Mean	26.38		7.0	9.0	16.0
SD	0.06		0.00	0.00	0.00
1	26.62	24:0	9	8	17
2	26.48	24:0	9	9	18
3	26.64	24:0	9	8	17
4	26.60	24:0	9	8	17
Mean	26.59		9.0	8.3	17.3
SD	0.07		0.00	0.50	0.50
1	20.37	0:24	8	13	21
2	20.24	0:24	8	13	21
3	20.32	0:24	8	13	21
4	20.35	0:24	8	13	21
Mean	20.32		8.0	13.0	21.0
SD	0.06		0.00	0.00	0.00
1	20.46	12:12	8	12	20
2	20.46	12:12	7	12	19
3	20.37	12:12	8	11	19
4	20.24	12:12	8	11	19
Mean	20.38		7.8	11.5	19.3
SD	0.10		0.50	0.58	0.50
1	20.48	16:8	9	11	20
2	20.67	16:8	8	11	19
3	20.52	16:8	9	10	19
4	20.37	16:8	8	12	20
Mean	20.51		8.5	11.0	19.5
SD	0.12		0.58	0.82	0.58
1	20.00[a]	24:0	8	10	18
2	20.00[a]	24:0	8	10	18
3	20.00[a]	24:0	8	12	20
4	20.00[a]	24:0	8	11	19
Mean	20.00		8.0	10.8	18.8
SD	0.00		0.00	0.96	0.96

Actual temperatures in larval rearing containers are reported by chamber (see Materials and Methods for details).
[a]Thermocouple malfunction. Set growth chamber temperature used.

However, the data shown in this study are inconclusive as to whether light is affecting *P. regina* in the larval stage or the pupal stage since the slopes of the linear regressions shown in Figure 6 are quite similar. *P. regina* reared in total darkness (0:24 [L:D] h) at 20°C increased their development time in both the larval and pupal stages compared with full light (24:0 [L:D] h). Nabity et al. (2007) discussed the issue of gating as an influence on emerging flies, where in previous studies, fly activity appeared strongly correlated with the onset of dawn (Greenberg 1991). An emergence gate is the period of time in a circadian cycle that corresponds to a light event when insects emerge as adults (Beck 1980). *Drosophila pseudoobscura* (Frolova) has an emergence gate of 6 h after dawn, and flies that complete development outside that gate will not emerge until the following permissive gate in the 24 h cycle (Beck 1980). Under this assumption, pupal duration should increase when reared in complete darkness, and emergence should be more variable when reared in constant light due to the lack of a stimulus
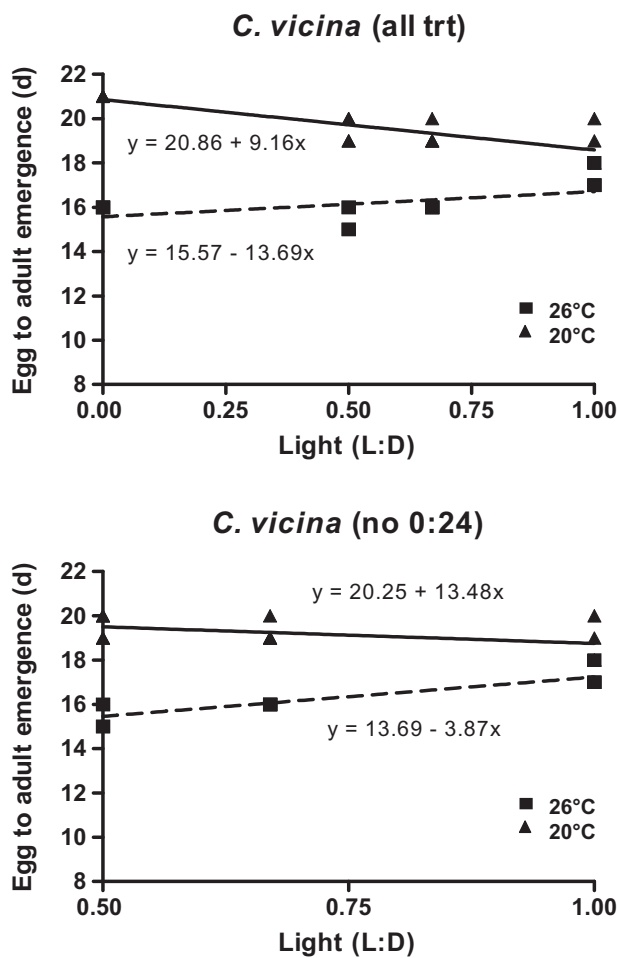
Fig. 4. Linear regression of *C. vicina* development (d) from egg to adult using rearing container temperatures showing regression slopes for all treatments (all trt) and without the 0:24 (L:D) h treatment. $R^2 = 0.6880$ (20°C); $R^2 = 0.3197$ (26°C).

Table 4. ADD for *P. regina*, *C. macellaria*, and *C. vicina* reared at set-chamber temperatures of 20°C and 26°C under the four light:dark (L:D) treatments

L:D	*P. regina*		*C. macellaria*		*C. vicina*	
	20°C	26°C	20°C	26°C	20°C	26°C
0:24	197.94	222.42	[a]	167.48	316.04	350.68
0:24	206.96	238.23	[a]	166.54	313.29	352.01
0:24	191.19	236.61	194.53	168.48	315.01	351.34
0:24	189.68	236.36	220.75	167.08	315.69	350.10
Mean	196.44	233.41	207.64	167.40	315.01	351.03
12:12	182.82	238.17	175.20	164.92	303.60	329.52
12:12	183.53	171.81	174.61	163.79	289.27	350.46
12:12	167.59	203.70	172.64	183.61	287.43	348.47
12:12	176.15	205.49	178.27	185.55	284.80	329.64
Mean	177.52	204.79	175.18	174.47	291.28	339.52
16:8	176.42	186.38	169.26	184.06	304.12	346.69
16:8	181.23	[a]	177.18	179.24	293.45	345.10
16:8	179.77	[a]	155.84	196.35	290.41	347.61
16:8	189.38	[a]	146.91	183.02	301.85	346.46
Mean	181.70	181.70	162.30	185.67	297.46	346.47
24:0	[a]	253.13	167.97	161.18	[a]	371.19
24:0	[a]	252.43	166.09	163.03	[a]	389.21
24:0	[a]	252.39	165.39	184.25	[a]	371.51
24:0	[a]	251.73	162.65	182.26	[a]	370.80
Mean		252.42	165.53	172.68		375.68
Total mean	185.47	223.05	174.85	175.18	301.25	351.99

[a]Thermocouple malfunction.

for stage transitions (Skopik and Pittendrigh 1967, Pittendrigh and Skopik 1970, Nabity et al. 2007). Figure 6 shows the linear regression slopes of *P. regina* for the development from pupa to adult. The data show that pupal duration actually decreases with constant darkness (0:24 [L:D] h) and that constant light (24:0 [L:D] h) increases pupal development. This suggests that stage transitions did not correlate to the onset of photophase.

C. vicina developed from egg to adult the fastest under cyclic light (12:12 [L:D] h) only at 26°C. Development from egg to adult and pupa to adult both showed significant effects of temperature, light, and the temperature by light interaction, but development from egg to pupa did not show significant effects of temperature. The absence of significance in the temperature term is likely due to experimental resolution. There is a temperature effect, but it appears that *C. vicina* is more sensitive to light rather than temperature across the temperature range tested. This would make sense due to the fact that if light is a diapause trigger, the flies would not be responding to temperature while in diapause anyway. In the temperate region of the United States, *C. vicina* is considered a cool-weather species, with increasing abundance seen in early spring and late fall. *C. vicina* is able to respond differently to light than *C. macellaria* and *P. regina* due to the fact that *C. vicina* will diapause given the proper environmental stimuli (Vaz Nunes and Saunders 1989, Vinogradova and Zinovjeva 1972). Diapause in *C. vicina* is under dual control where maternal inheritance determines the inclination to diapause via short days but only at temperatures below 15°C (Vinogradova and Zinovjeva 1972; Vaz Nunes and Saunders 1989; Nesin et al. 1995; McWatters and Saunders 1996, 1997; Saunders 2002; Vinogradova and Reznik 2002). Dispersing, third instars will enter a shallow diapause, given short days and temperatures below 15°C at the beginning of larval wandering (Saunders 1997, Saunders 2002, Fremdt et al. 2014). Conversely, larvae will not diapause and proceed to the next generation of flies through maternal exposure to long days (Saunders 2002). In all the experiments, no larvae of *C. vicina* entered diapause or exhibited any period of aestivation. Figure 7 illustrates the linear regression slopes for the development (d) of *C. vicina* from egg to pupa and pupa to adult. If we compare the linear regression slopes for the development of *C. vicina* from egg to pupa with pupa to adult and exclude the 0:24 (L:D) h treatment, we see that light had almost no effect on the development rate of the larvae at 20°C. However, at 26°C, we see significant differences in development rates, suggesting that light is affecting the larval stage. It is possible that like *C. macellaria*, light is affecting larval dispersal. It is also possible that *C. vicina* is able to adjust their metabolic rate accordingly to the duration of light, thus conserving energy.

Both *C. macellaria* and *P. regina* developed fastest under cyclic light but spent more time developing under cyclic light at the cooler 20°C temperature. This temperature by light interaction shows us that the effect on development rate is therefore more pronounced at lower temperatures. There was a temperature by light interaction seen in the experiments with *C. vicina*; however, *C. vicina* developed faster under cyclic light at the warmer 26°C temperature only. Previous research by Nabity et al. (2007) showed similar results with *P. regina*, indicating that the larval development of *P. regina* is significantly affected by the photoperiod. *C. macellaria* appears to respond in the same manner. Based on these results, it is possible that a temperature of 20°C is diagnostic for warm-weather species. For cool-weather species like *C. vicina*, temperatures less than 20°C may be more diagnostic since the results differed from *P. regina* and *C. macellaria*. To further demonstrate the fact that larval growth rates are affected by photoperiod and that the effect is more pronounced at cooler temperatures, the second experiment was conducted with *P. regina* at 16°C and 18°C under 12:12 (L:D) h, 16:8 (L:D) h, and 24:0 (L:D) h light regimes. The results showed the same trend of the effect of light being magnified at lower temperatures. The development of the larvae was affected by photoperiod. The R^2 values for some of the 26°C treatments are low, despite the points on the regression appearing to have a strong fit with the line. This is because the
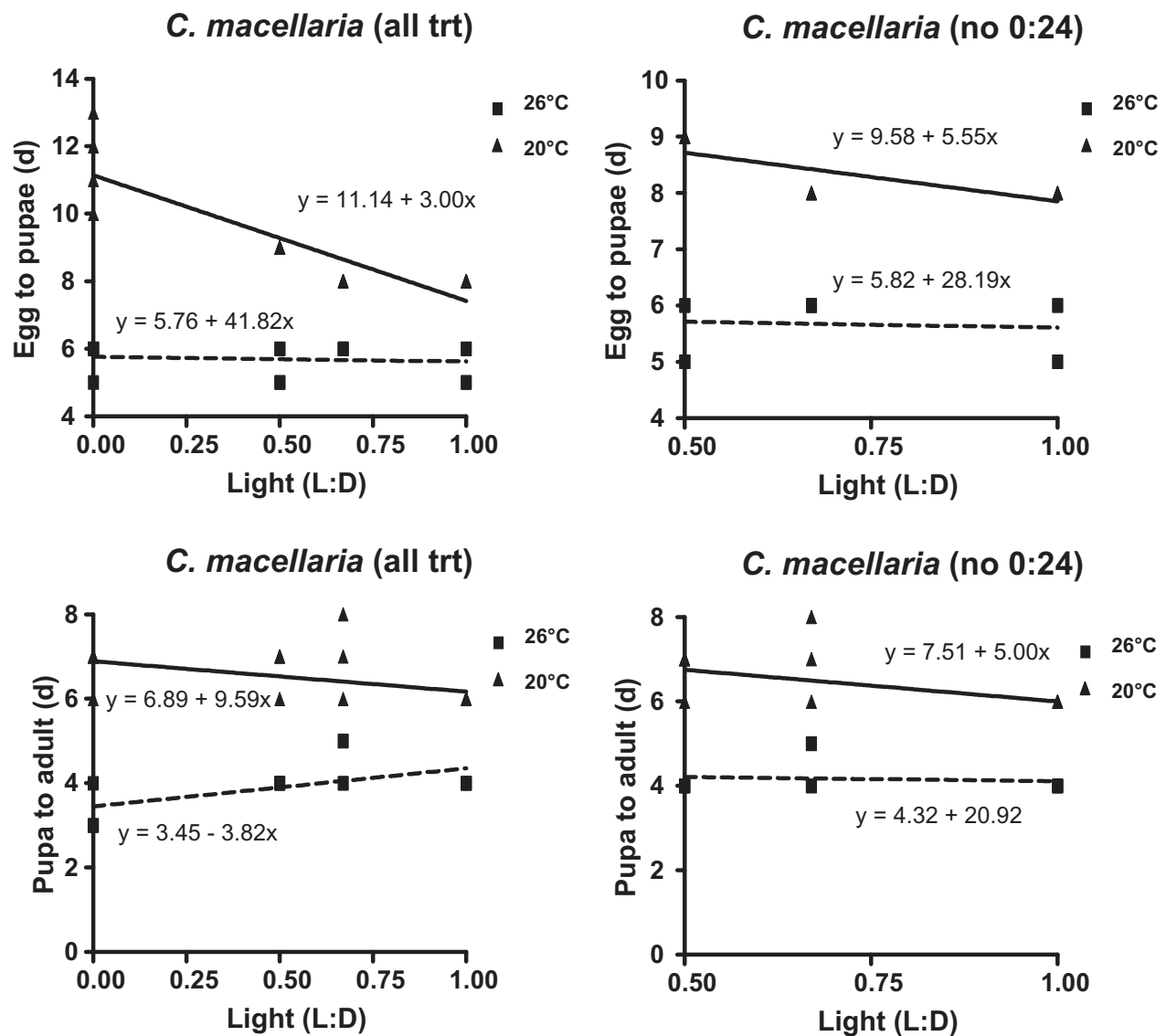
C. macellaria (all trt)

C. macellaria (no 0:24)

C. macellaria (all trt)

C. macellaria (no 0:24)

Fig. 5. Linear regression of *C. macellaria* development (d) from egg to pupa and pupa to adult using rearing container temperatures showing regression slopes for all treatments (all trt) and without the 0:24 (L:D) h treatment.

actual slope of the line is quite low and thus indistinguishable from zero (GraphPad Prism 5, GraphPad Software Inc. 2003).

It is important to consider the phenology of each species or seasonality, as well as geographic region when trying to determine the most appropriate light regime to which it is correlated. Photoperiod is the cue for seasonal synchrony in temperate regions, where the interaction of photoperiod and temperature determines phenology (Turchetto and Vanin 2010). This consideration is especially pertinent when estimating the PMI. When estimating the PMI, it is necessary to use the correct photoperiod corresponding to that geographic region. A PMI using species data collected from *P. regina* in Alaska in December will likely vary from a PMI using data from Florida in December. It is also entirely possible that differences will be observed based on genetic variation within species. Molecular techniques for age estimation of blow flies have focused on expression of diapause-specific heat-shock proteins, as well as other genes (Tachibana et al. 2005, Tarone et al. 2007, Tarone and Foran 2011, Concha et al. 2012, Boehme et al. 2013, Fremdt et al. 2014). Photoperiodic responses, including the length of the critical photoperiod and the proportion of the population entering diapause, are evolved characters that match the local conditions of photoperiod and temperature in the area from which the population was derived (Saunders 2002). If photoperiod is indeed maternally directed, and if

P. regina has adapted through generations to a 12:12 (L:D) h cycle, it may be inappropriate to conclude that the flies develop fastest at 16:8 (L:D) h. In addition, it is possible that the flies may be able to alter their metabolic rates to adjust to a specific light regime. We can also consider what to expect from flies' behavior in the tropics. Saunders (2002) discusses the idea that insects in the tropics do exhibit seasonal cycles of dormancy and metabolic activity, but it could not be concluded whether or not the dormancy is true diapause or rather a state of quiescence. Research using flesh flies (Sarcophagidae) was conducted in Nairobi, Kenya (1°S) and Belem, Brazil (1°S), which experience annual changes in day length of only ± 7 min, and it was determined that the induction of diapause depended on the temperature and not the photoperiod, where cooler temperatures during larval development were the most effective stimuli (Saunders 2002). Whether or not the same phenomenon is seen in blow flies is unclear. Of the three species tested in these experiments, *C. vicina* is the only species known to diapause as larvae based on previous literature (Vaz Nunes and Saunders 1989; Nesin et al. 1995; McWatters and Saunders 1996, 1997, 1998; Vinogradova and Reznik 2002). *P. regina* diapauses and overwinters in temperate regions as adults (Stoffolano 1973; Stoffolano et al. 1974; Stoffolano 1975; Greenberg and Stoffolano 1977). Other blow fly species know to diapause include *Lucilia sericata* (Meigen), *Lucilia caesar* (L.), and *Pr.*
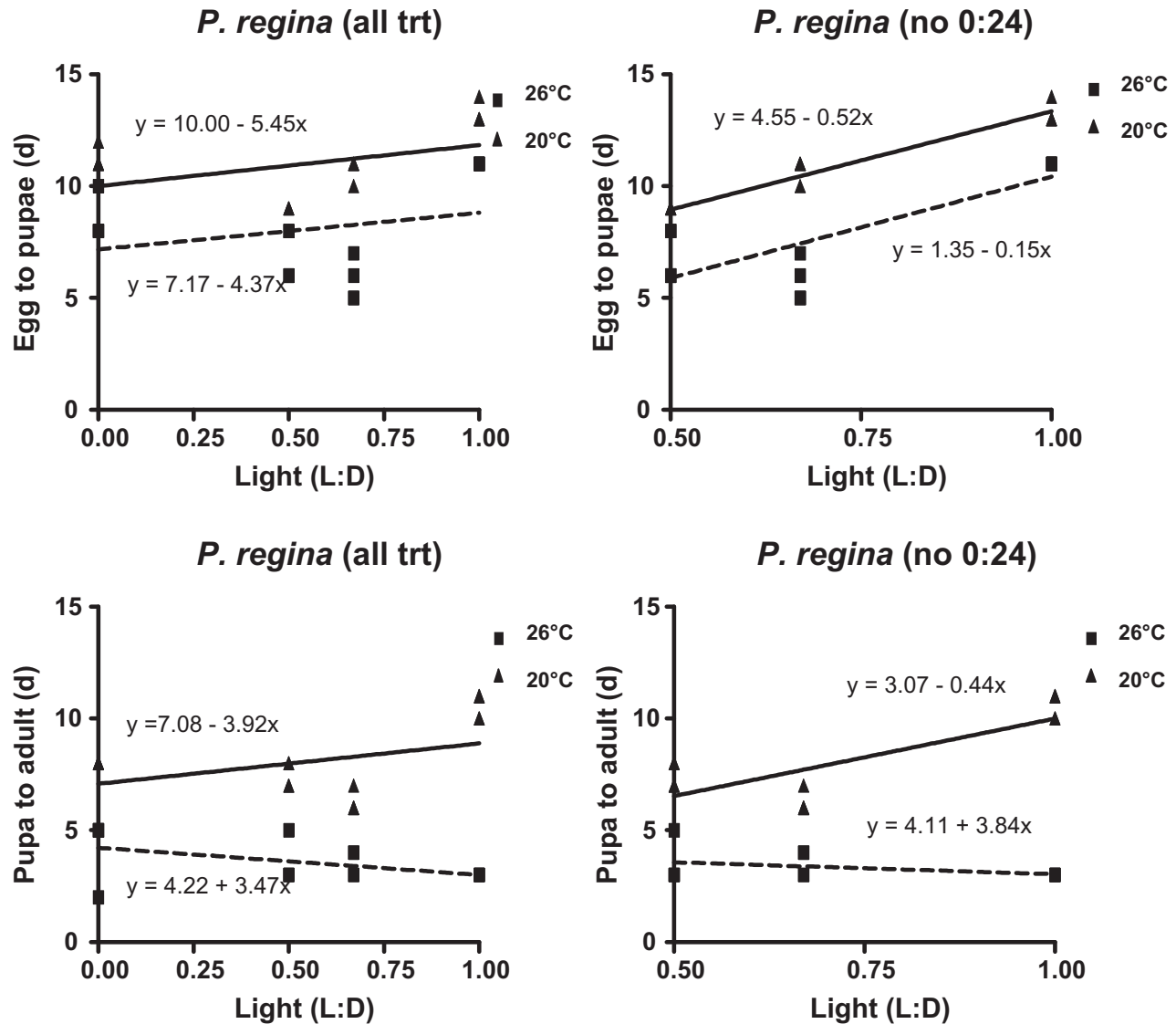
Fig. 6. Linear regression of *P. regina* development (d) from egg to pupa and pupa to adult using rearing container temperatures showing regression slopes for all treatments (all trt) and without the 0:24 (L:D) h treatment.

terraenovae (Cragg and Cole 1952, Fraser and Smith 1963, Ring 1967, Shiga et al. 2003, Tachibana and Numata 2004). *Cochliomyia homini-vorax* (Coquerel) does not diapause (Krafsur 1985), and it is unknown if *C. macellaria* will diapause under certain stimuli. Parish (1945) reported that *C. macellaria* apparently will not overwinter in Texas. Saunders (2002) reported five strains of *C. vicina* with a latitudinal range (S→N) of 36–65°N, and a critical day length of 15 h that increases by 1 h for every 4.8° of latitude. *C. vicina* exhibits a higher incidence of larval diapause under very long photophases, but in certain instances of overcrowding, under-sized larvae from eggs laid by short-day adults at 11°C will skip diapause and pupate (Saunders 1997, Saunders and Cymborowski 2003). Larvae of *L. caesar* show sensitivity to photoperiod in all instars and that no particular stage is one more sensitive compared with another (Ring 1967).

As stated by Nabity et al. (2007), previously calculated PMI's may be more variable than previously recognized. This is primarily applied when estimating narrower PMI's. A wider PMI will already encompass increased variability and may not be affected by any adjustments made for the effects with regard to light. Since many previous studies either did not report the experimental photoperiod or used 24:0 (L:D) h, it is difficult to determine the accuracy of any PMI using this data, as we have shown

that light affects each species in different ways. A PMI using constant light (24:0 [L:D] h) would represent an extreme situation and thus the PMI would be too slow. The results shown in Table 4 of the ADD for each treatment from egg to adult further support the conclusion that light affects development rate and that development rate is fastest under cyclic light at 20°C compared with the 0:24 (L:D) h. The differences in ADD vary among and in between the light regimes, but some more pronounced than others. *P. regina* showed a large difference in ADD at 26°C between 0:24 (L:D) h and 16:8 (L:D) h but much less at 20°C, whereas *C. macellaria* showed greater differences between 0:24 (L:D) h and 16:8 (L:D) h at 20°C as opposed to the 26°C treatments. *C. vicina* showed very small differences in ADD at 26°C between 0:24 (L:D) h and 16:8 (L:D) h, but the largest difference was between 24:0 (L:D) h and 16:8 (L:D) h. Differences like these may lead to underestimated errors and inaccurate PMI determinations (Nabity et al. 2007). The observed differences and variation seen with development and ADD reinforce the need to include the photoperiod when analyzing entomological evidence used in criminal investigations since assigning the incorrect light regime may distort or skew the development data.

At the time this study was conducted, it represented the most comprehensive look at how photoperiod can influence the development
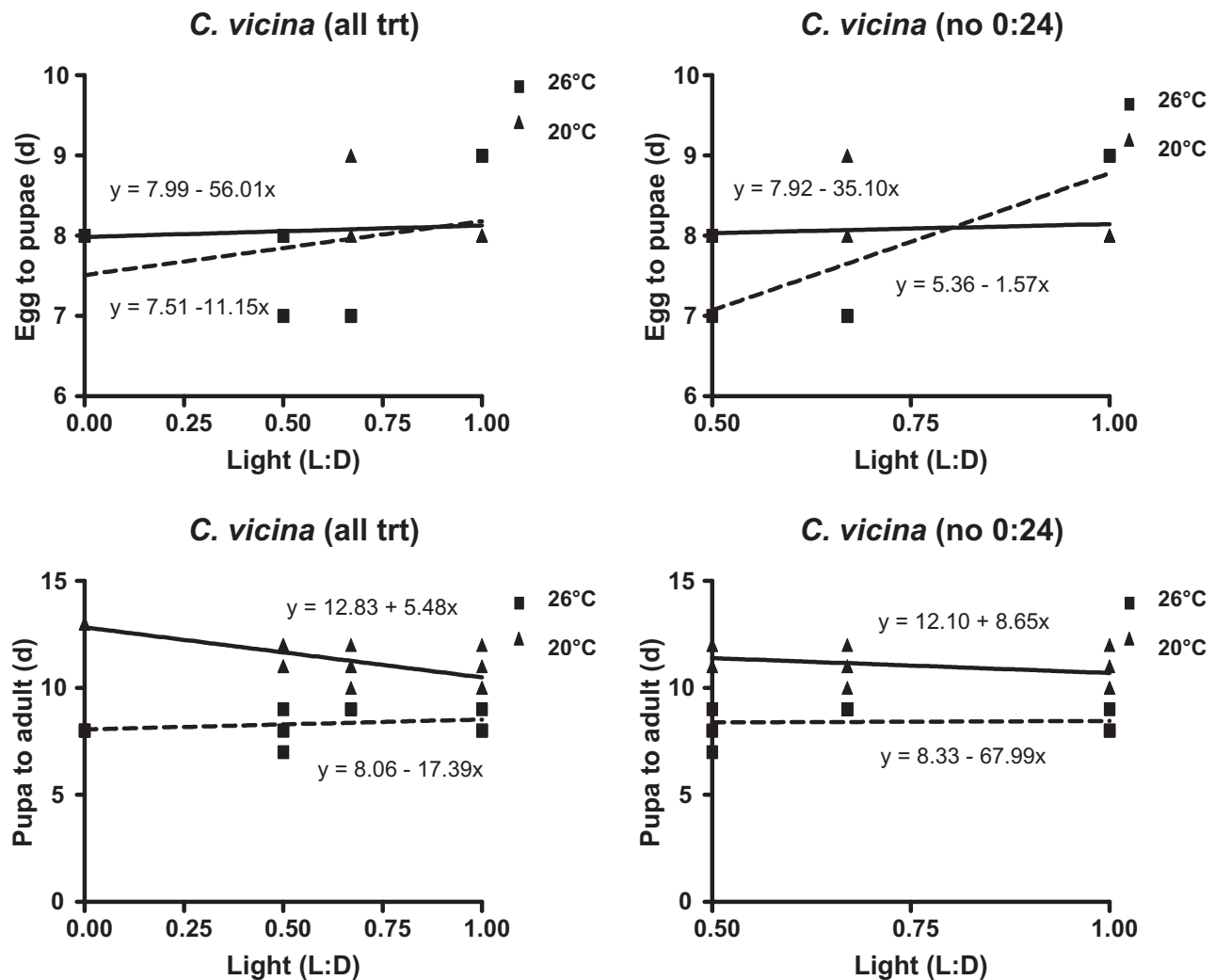
C. vicina (all trt)

C. vicina (no 0:24)

C. vicina (all trt)

C. vicina (no 0:24)

Fig. 7. Linear regression of *C. vicina* development (d) from egg to pupa and pupa to adult using rearing container temperatures showing regression slopes for all treatments (all trt) and without the 0:24 (L:D) h treatment.

rates of three of very common forensically important blow flies to the point where estimates of the PMI can have significant variation based on photoperiod alone. The data suggest that the light regime affects various species differently, where some slowed development under certain light conditions, while speeding up under others. These experiments could easily be repeated by future investigators to create a more inclusive set of empirical data on photoperiod's effect on blow fly development rates. Environmental factors such as the reported temperature, as well as photoperiod that affect the development rates of forensically important species of insects should be considered for increased accuracy when using entomological evidence in criminal investigations.

Acknowledgments

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