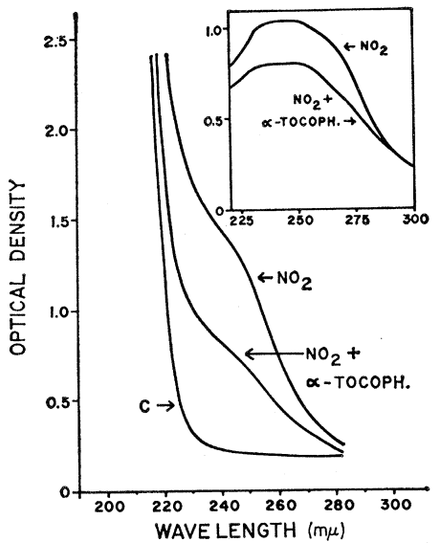

Fig. 3. Ultraviolet spectra for lung lipids (0.4 mg/ml) after inhalation of NO_2 (concentration 1 ppm) 4 hours daily for 6 consecutive days with respective controls. Inset: difference spectra obtained between peroxidized lipid and an equivalent quantity of nonperoxidized lipid. *NO_2*, exposed to nitrogen dioxide without α-tocopherol supplement; *NO_2 + α-tocoph.*, exposed to nitrogen dioxide with α-tocopherol supplement; *C*, exposed to ambient air and without α-tocopherol supplement.

also accompanied by characteristic structural change. We believe that this is the first time that chemical changes of this nature in animals exposed to such low concentrations of nitrogen dioxide have been reported.

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Circadian Oscillation Controlling Hatching: Its Ontogeny during Embryogenesis of a Moth

Abstract. *Populations of eggs of the moth* Pectinophora gossypiella *develop a circadian rhythm of hatching activity under certain circumstances. This rhythm derives from circadian oscillations in each egg, which can be initiated or made synchronous by steps or pulses of either light or temperature, but only if these signals are administered after the midpoint of embryogenesis. Correlations between the development of the oscillation, appearance of a pigment, sensitivity to photoperiodic induction, and a possible effect of light on growth rate are discussed.*

The moth *Pectinophora gossypiella* is subject to a photoperiodically inducible diapause in the fourth larval instar. It is easily reared on an artificial medium in the laboratory and has consequently become a useful object for the study of the photoperiodic time-measurement. In attempting to determine how circadian oscillations effect the measurement of night length in this species, we have recently sought and found three easily studied circadian rhythms in the egg, pupa, and adult. We here describe the rhythm found in the egg and report that the (cellular) oscillation responsible for the rhythm does not develop until midway through embryogenesis.

Stocks are maintained according to methods developed and described by Adkisson (*1*). They are kept at 26°C on a light-dark cycle of 14 hours of light (about 220 lumens of cool white fluorescent light per square meter) and 10 hours of dark (LD 14 : 10). Eggs are collected from parents kept in lucite boxes (13 by 19 by 9 cm) and fed sugar water (20 percent sucrose). A fine-mesh copper screen covers a square opening (8 cm^2) in the ceiling. Black velour paper (velour surface down) pressed against the screen provides an attractive surface for oviposition. Each night several thousand eggs are laid on the paper. They are oval (about 0.5 mm long and 0.25 mm wide), straw-colored, and only slightly opaque. At 20°C, embryogenesis lasts about 9 days, after which the small, first instar larva hatches.

The time of hatching in samples of the eggs collected in one night is assayed as follows. Egg-laden paper (300 to 400 eggs) is pasted to a carrier which in turn is placed on a fraction-collector device consisting of a lucite disk (33 cm in diameter) in which there are 24 slots, near the periphery, each surrounded by an adhesive cement (*2*). The egg carrier fits into these slots. Newly hatched larvae promptly explore the immediate neighborhood and are trapped in the adhesive. Every hour the egg carrier is automatically transferred to the next slot. Each day the lucite disk is replaced, and the trapped larvae are counted.

The night's egg-laying primarily occurs in the first few (about 4) hours (*3*). This small initial asynchrony in development within the sample is amplified throughout the next 10 days by variation in individual developmental rates and hatching behavior. Hatching of populations raised in a constant temperature and constant light (LL) is aperiodic, occurring over about 52 hours; the median developmental time in LL at 20°C is about 244 hours (Fig. 1A). A similar aperiodic distribution of hatching occurs in samples raised at 20°C in constant dark (DD), but both the median developmental time, about 275 hours, and the range of the distribution are much greater (Fig. 1A). The faster development in constant light may reflect a genuine effect of light on growth rate but we cannot yet confidently exclude the possibility that the weak illumination from the water-jacketed fluorescent lights slightly increases the temperature of the eggs. Light does have a direct action on the egg, promoting hatching when the animal is otherwise ready (see below). We believe that the reduced variance on developmental time in constant light is caused by that direct action.

In a light-dark cycle of LD 12 : 12, hatching is partitioned into discrete packets, one per day, with the median of each distribution coming soon after dawn (Fig. 1B). The eggs in the four cultures shown were laid in the same 10-hour night period; the phase of the four light regimes is staggered by 6-hour intervals. It is as though a portion of each light period constituted a "gate" (*4*) in which hatching could occur. The earlier the first gate relative to the ungated distribution in LL, the smaller is the number of eggs ready to

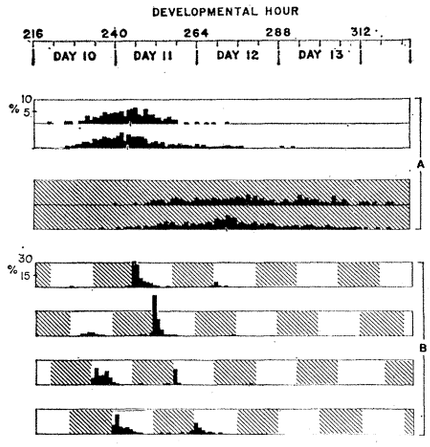

Fig. 1. (A) Hourly hatching of larvae from replicate populations (300 to 400 eggs) of *Pectinophora gossypiella* reared at 20°C in constant light [220 lumen/m² (upper)] or in constant dark (lower). (B) Distribution of larval hatch at 20°C in four regimes of LD 12 : 12 which differ by 6 hours in the time of the daily onset of light. Shading indicates dark.

exploit the gate; the later the first usable gate (cultures 2–4), the greater is the number ready to exploit it.

The fact that this is an entrained circadian rhythm is made clear by Fig. 2. Most eggs hatch on the 10th day of development; hatching of a small fraction is delayed to the following day. When the light cycle is discontinued, hatching is delayed and spread over 4 days, but it is still partitioned into discrete peaks separated by a circadian (nearly 24 hours) interval.

The oscillation responsible for this rhythm has another property characteristic of circadian oscillations, namely innateness. A single transition from constant light to constant dark (Fig. 3, populations 12 to 24); a single light pulse (15 minutes, 220 lumen/m²) (Fig. 4A, lower line); and a single 12-hour temperature pulse of 28°C (Fig. 5B) will induce circadian rhythmicity in populations that would otherwise be aperiodic. An autonomous oscillatory mechanism is clearly innate; the single signals, none of which give information on period, initiate the oscillation synchronously in all of the eggs.

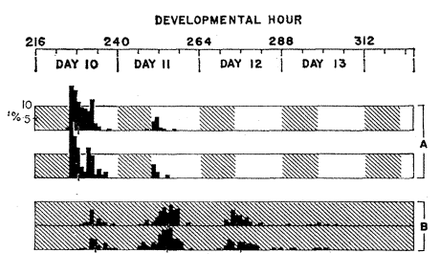

Fig. 2. Circadian rhythmicity of hatching at 21°C, when replicate egg populations are reared and continued in LD 14:10 (A) or placed in constant dark after 9 days in LD 14:10 (B). Shading indicates dark.

We noticed that signals applied in the earlier half of development had no effect on the distribution of hatching (Fig. 3). A systematic study revealed that (i) transitions from constant light to constant dark (ii) single 15-minute pulses of light (220 lumen/m²), and (iii) sequential cycles of LD 12 : 12 failed to induce a rhythm when applied during the first 5 days of development; all were effective inducers from the middle of day 6 onward.

Figure 3 shows the distribution of hatching in 24 egg populations all raised in LL and transferred to DD at systematically later times (5½-hour intervals) throughout development (culture 1 at 82 hours, culture 2 at 87.5 hours, and so on). Clear rhythmicity is only induced when the transition from LL to DD occurs on or after 132 hours, about midway through development. The phase of the rhythm is dictated by the time of the transition which initiates it (t_i).

A single 15-minute pulse is ineffective at 104 hours but is effective at 132 hours (Fig. 4A); and Fig. 4B gives comparable information for a light-dark cycle (LD 12 : 12) (light 9 a.m. to 9 p.m.) discontinued at the end of day 4, and at the end of day 7.

The discrete onset of susceptibility to initiation midway in day 6 is clear (Fig. 3). The absence of a rhythm in populations 1 through 9 cannot be attributed to its having been damped out subsequent to successful initiation; signals on day 6 give clear peaks on day 12; here the oscillation has not been damped in 6 days, and the failure to find a distinct peak on day 11 following a signal on day 5 cannot therefore be attributed to damping.

Failure of these diverse light signals before 132 hours of development to initiate rhythmicity leaves two very different interpretations open: (i) the oscillatory mechanism underlying the rhythm does not develop until that time; and (ii) the mechanism is present from the outset, but the photoreceptor system which couples it to the light cycle does not develop before 132 hours. The second alternative appeared

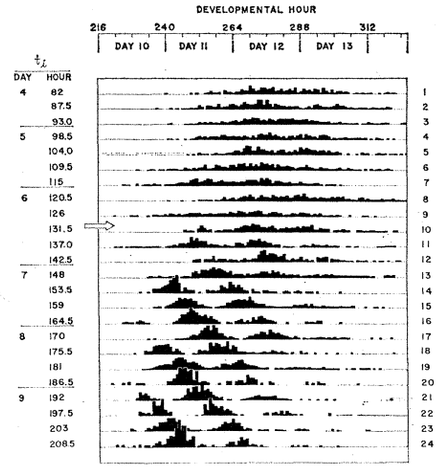

Fig. 3. Normalized distribution of larval hatch in continuous dark from 24 populations of eggs (numbered at right) which were reared at 20°C in constant light, 220 lumen/m², and systematically shifted to constant dark at 5½-hour intervals, from hours 82 through 208.5. The time of the transition is indicated by t_i. Shading to indicate constant darkness has been omitted to avoid obscuring the details of the distributions.

plausible because at about this time a pinkish pigment appears in the head region of the embryo.

The second interpretation has, however, been excluded by experiments suggested by Dr. M. Menaker. In *Drosophila pseudoobscura* (*5*) and other species, circadian rhythmicity can be initiated in otherwise aperiodic populations by single temperature pulses. In this case, the presence or absence of specific receptor systems is of course not an issue in the induction of rhythmicity, nor is it likely that the action in resetting or initiating rhythmicity requires a specific temperature receptor system. We have found,

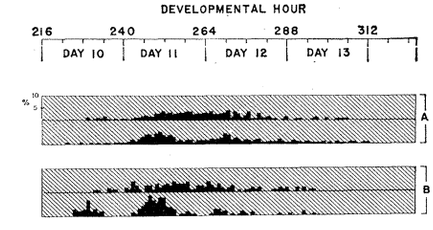

Fig. 4. (A) Distribution of larval hatch following a single 15-minute light pulse, 220 lumen/m², at 114 hours (upper), or at 132 hours (lower). Cultures at 20.5°C and in continuous dark before and after the light pulse. (B) Distribution of larval hatch from cultures reared in 12 hours of light and 12 hours of dark (upper) for four cycles, followed by constant dark; (lower) for seven cycles, followed by constant dark. Shading indicates dark.

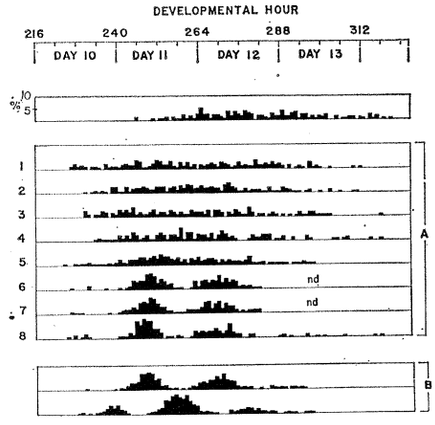

Fig. 5. (A) Effect of a single 12-hour temperature pulse (20°C/28°C/20°C) on the hatching of populations reared in continuous darkness. Numbers at left indicate day on which the pulse was given. Control (constant dark and constant 20°C) at top. (B) Distribution of hatching following a temperature pulse given on day 6 from 9 a.m. to 9 p.m. (upper), or from 9 p.m. to 9 a.m. (lower). Shading indicating constant darkness is omitted.

in *Pectinophora*, that a 12-hour temperature pulse induces rhythmicity when applied on day 6, or 7, or 8 but fails to do so on day 5 and earlier (Fig. 5A). Clearly the oscillator responsible for the ultimate timing of hatching, not its photoreceptor system, is what is lacking until the middle of the 6th day.

It seems probable to us that what remains undeveloped before day 6 is a specific element in the central nervous system which controls hatching. The absence of this specific "oscillator" before this day surely cannot imply a more generally inadequate complexity to sustain any circadian oscillation. Many protistan cells sustain them, and there is the remarkable and unique case (*6*) of circadian phase being transmitted from the mother through the egg of the Australian fruit fly *Dacus*. Kalmus (*7*) and Edwards (*8*) have reported the only other cases known to us of a circadian rhythm of hatching, but in neither case is it known that the responsible oscillation is present (that its phase can be set) before differentiation proceeds. Brett (*9*) reported that in *Drosophila* the phase of the rhythm of pupal eclosion can be set in early larval stages but not in the egg. The principal interest attaching to our observations is the potential they afford for further analysis of several correlations.

First, the fact that a circadian oscillation develops at about the same time that the pink pigment appears encourages the hypothesis that the pigment is involved in coupling the oscillation to the light cycle. That hypothesis can be tested by obtaining an action-spectrum for the oscillation's response to light, which can then be compared to the pigment's absorption.

Second, the photoperiodic induction of diapause in the fourth larval instar can be effected by treatment of the egg, a fact reported by Adkisson (*10*) and confirmed by us. Adkisson's data leave open the interpretation that such photoperiodic induction can, however, only be effected in the latter half of embryogenesis. Further data are needed before one can define the onset of photoperiodic inducibility as sharply as our data define the onset of the circadian oscillation.

Third, available data suggest that the effect of light on the rate of development, whether it be a genuine effect of light on growth rate or an indirect effect of temperature, is restricted to the latter half of development. The aperiodic distributions of hatching in cultures 1 to 10 in Fig. 3 show no clear tendency to be advanced the longer the light is on. This in itself renders implausible the hypothesis that increased temperature is involved. Among these populations there is variation in rate of development, measured by the onset of hatching or the median of the distribution. This is attributable to slight shifts in the temperature of the rearing boxes during the several months while the experiments were undertaken. Within each single experiment the agreement between populations was very close. For example, populations 1, 3, and 5 (a little slower) constituted one experiment; 7, 9, and 11 (a little faster) constituted another. It therefore remains possible, and testable, that the onset of the capacity to sustain a circadian oscillation is coincident with the onset of photoperiodic inducibility, and the onset of an effect of light, as such, on developmental rates.

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Vascular Smooth Muscle: Quantitation of Cell Thickness in the Wall of Arterioles in the Living Animal in situ

Abstract. *The wall of microarterial vessels, in the living animal* in situ, *was examined at magnification up to × 6500 on the television screen. The optical resolution af the cell components of the wall was sufficiently clear to permit image formation and splitting for permanent recording and quantitation. Thickness of single smooth muscle cells at rest was estimated to be 2.08 microns (S.D. ± 0.24 micron) and 2.78 microns (S.D. ± 0.59 micron) by two different approaches. Changes in cell thickness during activity were also recorded and quantitated.*

Control of blood flow and volume distribution by the microvessel depends primarily on the effector smooth muscle cell in the wall. Yet reliable information relative to the events of the contractile process of the muscle cell at this level of the circulation is scanty. Except for measurements of intracellular potentials in microvessels made in the living animal *in situ* (*1*), most of the data concerning the contractile process in vascular smooth muscle are derived from studies in vitro of tissue samples obtained from the larger segment of the circulatory system. The aim of the experiments reported here was to determine and measure a geometric variable in single smooth muscle cells in the wall of precapillary arterioles, both under steady state and during activity of the vessel, in the living animal *in situ*.

The experiments were carried out in arterioles of mesentery and skeletal