5, and then eluted with acetic acid (20 mM). This liberated chitinase in a single peak with constant specific activity (fig. 1). The specific activity of purified chitinase was about 20-fold higher than in the crude culture filtrate, indicating that about 5% of the total secreted protein of *Aphanocladium album* is chitinase.

Purified chitinase of *Aphanocladium album* exhibited a broad pH optimum of pH 5 with 50% of the activity remaining at pH 3 and pH 7 (data not shown). A standard curve relating the amount of product formed in 30 min to the amount of enzyme was established (fig. 2). It was found that this standard curve was linear up to a production of 1 µmole GlcNAc equivalents from 1.2 mg substrate, corresponding to the hydrolysis of almost 20% of the substrate.

Washed, boiled mycelium of wheat rust, *Puccinia graminis* var. *tritici*, was incubated with dialyzed culture filtrate of *Aphanocladium album* containing 60 nkat chitinase or with 60 nkat purified chitinase from *Aphanocladium album*. Both preparations liberated soluble chitin fragments from the rust cell walls. Interestingly, the crude dialyzed culture filtrate was about twice as efficient as was purified chitinase. This indicates that other, unspecified enzymes of the culture filtrate enhance the potential of chitinase for attacking the mycelium of *Puccinia graminis* var. *tritici*.

Discussion. Ultrastructural studies have provided evidence that the attack of many hyperparasitic fungi involves lysis of the mycelia of their hosts[4-8]. As discussed by Tsuneda and Hiratsuka[6], this might result from autolytic processes induced in the host or from the activity of cell-wall degrading enzymes of the mycoparasites. In the present work, we have asked the question whether a mycoparasite, *Aphanocladium album*, has the enzymatic equipment to lyse the cell walls of its host, *Puccinia graminis* var. *tritici*. Rust fungi have a chitin-containing cell wall[14,15]; we therefore concentrated on chitinase. Our data show that *Aphanocladium album* secretes large amounts of chitinase on a chitin-containing synthetic medium. A distinguishing feature of this chitinase is its standard curve (fig. 2): The relation of product formation to enzyme concentration is almost linear until 20% of the substrate is hydrolyzed. This is in contrast to most other chitinases examined[10,11], which lose efficiency much sooner, when only 1–2% of the substrate is consumed, as shown by the much more curved standard curves. A chitinase resembling that of *Aphanocladium album* has previously been purified from *Serratia marcescens*[16].

We have purified *Aphanocladium album* chitinase and shown that it efficiently hydrolyzes chitin from the cell walls of its host. Interestingly, the culture medium contains other, unspecified enzymes that render the attack of chitinase yet more efficient. In this context, we found that crude culture filtrate completely dissolved the germ tubes of UV-killed *Puccinia graminis* while purified chitinase lysed the germ tubes only partially (data not shown). We conclude that chitinase is one important element in the attack of *Aphanocladium album* on *Puccinia graminis* var. *tritici* but is not in itself sufficient to bring about effective lysis of its host.

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Two mechanisms in the biological clock of *Pieris brassicae* L.: an oscillator for diapause induction; an hour-glass for diapause termination

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Summary. Resonance experiments for photoperiodic termination of pupal diapause demonstrated that *Pieris brassicae* uses a night-measuring hour-glass mechanism. In previous work the same resonance technique for diapause induction revealed that photoperiodic time-measurement is a function of the circadian system. For the first time in a living organism it has been shown that the biological clock operates by means of an oscillator for photoperiodic onset of a phenomenon and according to an hour-glass system for photoperiodic termination.
Key words. Clock; circadian oscillator; hour-glass; diapause; *Pieris*.

The biological clock, measuring time with regard to photoperiodic phenomena in living organisms, is assumed to function according to two basic principles: either a circadian oscillator[1] or an hour-glass (or a combination of the two). Resonance experiments, in which one phase, either day or night, is kept constant while the complementary phase is varied so as to obtain cycles with a period (T) of 18 to 72 h, and experiments in which an extented night is systematically interrupted by a light pulse, have proved to be most effective in revealing the existence of a circadian component. The interpretation of resonance experiments is that if the photoperiodic clock incorporates a circadian oscillation (i.e., with an endogenous periodicity, τ, close to 24 h), the product of induction is observed to be high when T is close to τ or modulo τ (i.e. the two oscillating systems resonate) or low when T is not close to modulo τ (i.e. the two oscillating systems do not resonate). In night interruption experiments, alternate peaks and troughs of a photoperiodic effect, approximately 24 h apart, are interpreted as manifestations of an underlying circadian rhythmicity (of period τ). A circadian system has been shown to exist in the majority of insects[2-6], vertebrate[7-18], and plant[19] species studied. Similar experiments have failed to reveal obvious circadian periodicity in a smaller number of species (insects[2,3,20], a lizard[21] and plants[19]), and in some cases the hour-glass interpretation is considered more appropriate. Bünning[22] examined the effects of 30-min pulses in the nights of 24-h, 36-h and 48-h cycles on larvae of the cabbage white butterfly *Pieris*

brassicae. The results provided no evidence that the circadian system was involved. But, recently, experiments with long-period night interruptions and with resonance experiments for diapause induction have both indicated a clear circadian component in *Pieris brassicae*[23].

The facultative diapause of *Pieris brassicae* is manifested by pupae in response to short days experienced by the caterpillars. The photoperiodic termination of diapause by long days requires a longer treatment (2–3 months)[24,25]. Diapausing pupae arising from larvae reared under a photoperiod of LD 10:14 at 20 °C were kept under these same conditions up to the experiments. The photoperiodic regimes for diapause termination comprised a constant component (a photophase of 16 h or a scotophase of 8 h) and a complementary component of variable length to give cycles of 18 to 72 h (T) by increment of 2 or 4 h. For each experiment 50–100 pupae were tested at 20 °C.

The results presented in figure 1 clearly show that the breaking of diapause is not a periodic function of T. With a photophase of 16 h, emergence from diapause was obtained only with LD cycles of 16:4, 16:6, 16:8 and 16:10, i.e. whenever the duration of the scotophase was equal to or below 10 h. In the reverse experiment, all light regimes provoked termination of diapause. No periodicity was found. The positive response induced by very long photophases cannot be attributed to constant light as in *Pieris*, LL is incapable of breaking the diapause[25]. The results indicate that a period of 8 h of darkness had been measured and that the measurement can take place regardless of the period of the light cycle. The length of the 'night' is more critical than the length of the 'day'. The results of both experiments were inexplicable in terms of the circadian model and conform to a non-repetitive dark period interval timer or hour-glass. This result is quite surprising in that analogous experiments concerning the induction of diapause have shown the existence of a circadian component.

In *Drosophila auraria* and *Sarcophaga argyrostoma*, it has been shown that the response curves in resonance experiments may be either periodic or aperiodic depending on the temperature at which the experiments are conducted[26,27]. A lowering of the temperature (from 17 to 15 °C for *D. auraria* and from 22 to 18 °C for *S. argyrostoma*) suppresses the rhythmicity of the response and the results then conform to an hour-glass model. To verify the possible modifying action of temperature in *Pieris*, the same resonance experiments were performed at 25 °C and 15 °C. The curves obtained (not shown) were identical to those illustrated in figure 1. In *P. brassicae*, the hour-glass type of response is therefore not due to the absence of thermal conditions permitting resonance. It has been remarked[2,3], that a negative result in these resonance experiments does not necessarily signify the absence of a circadian oscillator, the expression of which may be masked by the conditions of the experiment. However, the persistence of the response at different temperatures in experiments which, in the case of caterpillars, clearly reveal the involvement of a circadian system provides strong evidence that pupae of *Pieris* use an hour-glass rather than a circadian mechanism to measure photoperiodic time at the moment of breaking their diapause. Within the range from 15° to 25 °C – which occurs naturally during spring when diapause termination of *Pieris* is governed by long photoperiods – the temperature cannot affect the expression of the photoperiodic clock.

The response to the photoperiod, in the majority of species, appears only after several inducing photoperiodic cycles. The necessity of summation of periodic signals has led to the concept of a photoperiodic counter[28,29]. On the basis of previous experiments including a fixed scotophase, the number of signals necessary to reactivate 50% of diapausing pupae was determined for each cycle (fig. 2). The number of signals varies according to the period of the inducing cycle. The values obtained fall into two groups for each of which a linear regression line can be drawn. One regression line links the data obtained for cycles equal to or below 24 h, while the other joins those corresponding to cycles exceeding 24 h. The two regression lines intersect at T = 24 h. An increasing number of signals is required as the cycle diminishes below 24 h, the coefficient of augmentation being substantial.

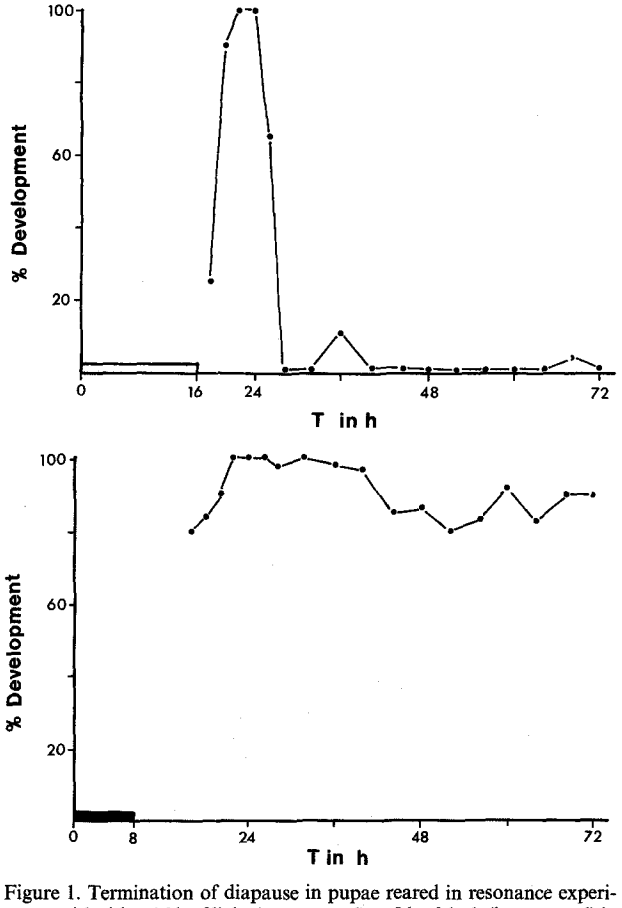

Figure 1. Termination of diapause in pupae reared in resonance experiments with either 16 h of light (upper panel) or 8 h of dark (lower panel) in cycles of up to 72 h.

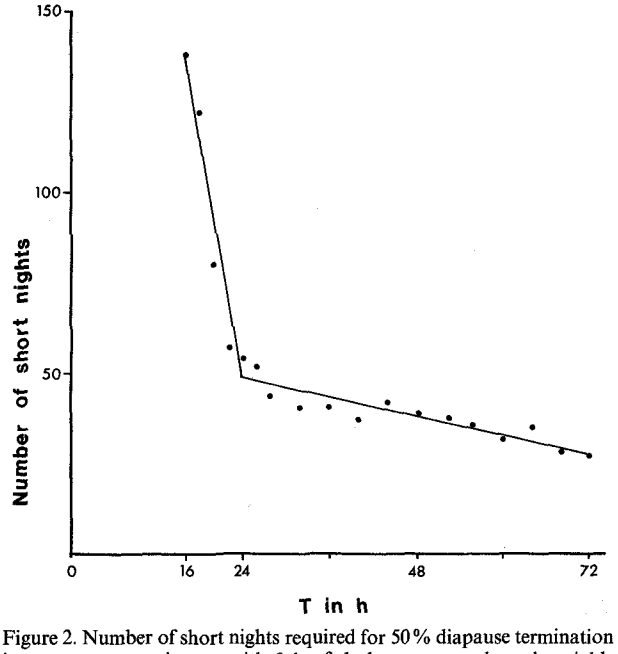

Figure 2. Number of short nights required for 50% diapause termination in resonance experiments with 8 h of darkness per cycle and variable periods of light.

Above 24 h the values diminish slightly as a function of T. The critical number of short night signals depends on the light regime. The clock-counter system works better with very long cycles. Below 24 h it loses its efficiency. If there were a circadian element in the system (either for clock or for counter), a higher efficiency would be expected as one approaches the natural period of 24 h.

The measure of time provided by the Zeitgeber is determined by a circadian system for the induction at the larval stage while it is apparently accomplished according to an hour-glass model for diapause termination in the pupa. This change in operation during development is indicated here for the first time. Metamorphosis in holometabolous insects involves the almost total restructuring of the tissues and organs, including the central nervous system. Two situations may occur: either there is a single clock to control entry into and exit from diapause, and metamorphosis provokes a change in its operation, or induction is controlled by a larval clock operating with a circadian oscillator and diapause termination by another, anatomically different pupal clock, working according to the hour-glass model.

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Oral melatonin produces arrhythmia in sparrows

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Summary. House sparrows, *Passer domesticus,* exhibit circadian rhythms of perch-hopping behavior. The rhythm was abolished by ad libitum administration of melatonin in the drinking water.
Key words. Passer domesticus; circadian rhythm; arrhythmia; melatonin; perch-hopping behavior.

Sparrows exhibit daily cycles of perch-hopping activity. The cycles persist in dim constant light (e.g. 40 lux) or in constant dark (DD, 0 lux) with period lengths close to 24 h and thus constitute a circadian rhythm. The circadian rhythm of perch-hopping that persists in DD is abolished by pinealectomy[2,3], lesions of the suprachiasmatic nuclei[4] or melatonin in implanted capsules[5,6]. In the experiments shown here we measured sparrows' perch-hopping behavior while we a) gave ad libitum access to water containing melatonin (lg/l) to discern whether oral melatonin could affect their rhythms, or b) implanted sparrows with 50 mm Silastic capsules containing melatonin in an attempt to replicate the prior result[5,6].

Sparrows were caught in the environs of Philadelphia. Trapped birds were maintained in a stock population in indoor aviaries (1.8 × 1.8 × 2.7 m) in LD12:12 (lights-on 6am Eastern Daylight Savings Time). Four male and 20 female sparrows contributed 48 records which form the data reported here. To record locomotor activity we placed a single sparrow in an isolation chamber. The chamber was a wooden box, painted black, which contained a cage. Two perches connected to microswitches made a single record of locomotor activity registered with Esterline Angus event recorders. The locomotor records of the sparrows were cut, pasted, and reduced photographically. Food and water was provided to the sparrows ad libitum. Light sources in the tops of the individual boxes made it possible to impose timed lighting programs on the birds. The light intensity in the boxes was 800 lux. The boxes were kept in rooms in which 90 db white noise prevented vocal interactions among the birds.

Melatonin was administered in Silastic capsules to 5 sparrows for portions of their recording periods. Capsules were made in a manner identical to those used by prior investigators – 50 mm capsules were implanted which, according to their estimates, release 40 µg of melatonin per day[5,6]. Implantations (i.p.) and removals were made under Equithesin anesthesia and involved 30 min of light exposure and handling. Controls were implanted with capsules which did not contain melatonin.

In 2 of 4 sparrows that exhibited circadian rhythms before the capsule treatments, melatonin capsules produced arrhythmia within 24 h of implantation (table, A). The third bird exhibited a relatively short freerunning period (tau = 23.6 h) and the fourth bird's rhythm became less clear when the capsule was implanted so that the period length could not be accurately measured. When the capsules were removed, circadian perch-hopping rhythms reappeared within 24 h. There was no evidence that the phase of the re-emerging rhythms extrapolated back to the phase of the rhythms before melatonin (that is, melatonin did not mask