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Integrated management of the two-spotted spider mite, Tetranychus urticae: photoperiodism, UV sensitivity and GABA receptor

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Doctoral Dissertation

Integrated management of the two-spotted spider mite, *Tetranychus urticae*: photoperiodism, UV sensitivity and GABA receptor

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博士論文

ナミハダニ総合防除のための基礎研究：光周性，紫外線感受性および GABA 受容体

鈴木 丈詞

神戸大学大学院 自然科学研究科

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Integrated management of the two-spotted spider mite, *Tetranychus urticae*: photoperiodism, UV sensitivity and GABA receptor

Takeshi Suzuki

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Chapter 1

General Introduction

1 Integrated pest management (IPM)

To date, pest control has heavily relied on chemical agents since these are convenient and give immediate effects. However, the frequent use of chemicals often induces (1) resistance to pesticides in pest arthropods, (2) elimination of beneficial organisms, (3) pest resurgence, and (4) persistence of residues in environment.

For example, there is virtually no crop without mite pests, but phytophagous mites were regarded as in the status of secondary importance as pests in orchard before the 1940s (March, 1976). However, mite problems became more serious with the advent of modern pesticides and agricultural practices, and for the past few decades, phytophagous mites as a group have been rated among our most serious pests (Chant, 1966).

DDT (dichloro-diphenyl-trichloroethane) [4,4'-(2,2,2-trichloroethane-1,1-diyl)bis (chlorobenzene)] (IUPAC) and other chlorinated-hydrocarbon insecticides were found to be essentially ineffective against phytophagous mites. Not only were they ineffective, but also their application for insect control resulted in the increase of mite and aphid populations to serious pest status (March, 1976).

Such outbreaks of one-time secondary pests apparently result from a complex of causes; direct effects of the pesticides on predators, host plant physiology and pest fecundity, and altered agricultural management must have contributed to this situation (Bartlett, 1968; Dittrich et al., 1974; McMurtry et al., 1970; Huffaker et al., 1970).

Therefore, the implementation of integrated pest management (IPM)—an approach that employs a combination of existing pest management measures including timely application of small amounts of chemicals—is urgently needed. The Food and Agriculture Organization of the United Nations (FAO) defines IPM as follows: "the careful integration of a number of available pest control techniques that discourage the development of pest populations and keep pesticides and other interventions to levels that are economically justified and safe for human health and the environment." Next to the chemical control, the conventional maneuver of IPM is biological control. However, physical control has been relatively unexploited.

1.1 Photoperiodic control utilizing light-emitting diode (LED) technology

The light-emitting diode (LED) is a new source of light that is expected to replace conventional "vacuum system" light sources, e.g., incandescent lamps, fluorescent tubes, and high-intensity discharge lamps. LEDs have found their applications not only in pilot lamps, traffic signals, and digital display systems but also in agriculture, forestry, and fisheries as enhancers of luminous efficiency and color specificity. In particular, LED technology has been put to practical use in plant production systems based on its capacity to produce various monochromatic lights and to irradiate plants with light closely and uniformly. In arthropods, development, reproduction, diapause, behavior, and morphology are often regulated by the light environment in terms of photoperiod, light quality and intensity. Therefore, a utility of LED technology in controlling harmful arthropods can be expected.

Physical controls have been under-exploited but the manipulation of lighting sometimes provides effective measures. For example, yellow light repels noctuid moths in the orchard (Nomura et al., 1965). Although the cost per light output of LEDs is still higher than that of other light sources, LEDs can be useful in pest control by virtue of their spectral characteristics, flexible shape, and low emittance of heat radiation. Indeed, LEDs have been sometimes utilized for trapping insect pests (Burkett et al., 1998; Chu et al., 2003; Chen et al., 2004; Nakamoto and Kuba, 2004; Bishop et al., 2006; Hoel et al., 2007). Here, I propose the potential application of artificial lighting including LED technology in the control of agricultural arthropod pests in such a way that photoperiodic manipulation disturbs their life cycles.

Photoperiod is an accurate environmental signal by which organisms track the time of day and the passage of the seasons (Tauber et al., 1986). Therefore, photoperiodism is commonly adopted by great many organisms as a calendar for their life cycles. It determines the timing of diapause and the appearance of particular morphs in many cases. In insects and mites, diapause is an adaptation evolved to cope with unfavorable seasons. Diapause is so critical for the survivals of insects and mites that any disturbance in its timing or expression could give severe negative consequences. Accordingly, this could potentially be exploited as a part of IPM (Denlinger, 2008). If diapause is untimely induced or terminated, it can hinder pest population growth.

Cumulative data have shown that diapause can be prevented by photoperiodic manipulation in the tortricid moth *Adoxophyes orana* (Ankersmit, 1968; Berlinger and Ankersmit, 1976), the European corn borer *Ostrinia nubilalis* (Hayes et al., 1970, 1974b, 1979; Schechter et al., 1971), the codling moth *Cydia* (*Laspeyresia*) *pomonella* (Hayes et al., 1970, 1974a; Sáringer, 1982; Schechter et al., 1971), the tobacco budworm *Heliothis virescens* (Hayes et al., 1974a), the pink bollworm *Pectinophora gossypiella*

(Sullivan et al., 1970; Hayes et al., 1974a), the oak silk moth *Antheraea pernyi* (Hayes et al., 1974a), the rock pool mosquito *Aedes atropalpus* (Beach and Craig, 1979), and the turnip sawfly *Athalia rosae* (Sáringer, 1983). In *O. nubilalis* and *C. pomonella*, a high level of diapause prevention has been observed in the field in response to artificially extending the day length using fluorescent tubes or mercury vapor lamps (Hayes et al., 1970; Schechter et al., 1971). Diapause prevention by appropriately timed nocturnal light breaks in the field using fluorescent tubes has been successful in some cases (Hayes et al., 1974) but not in others (Hayes et al., 1970; Schechter et al., 1971; Berlinger and Ankersmit, 1976). Ankersmit (1968) reported that in the field a 2-min light pulse using fluorescent tubes prevents diapause in *A. orana*. Unfortunately, such measures have not been developed further since achieving sufficient light intensity in the field is economically prohibitive. However, Suzuki and Takeda (2009) suggest that LED technology can solve this problem on account of the following properties: (1) the energy consumption of LEDs is low and their life span is long, (2) high light intensity can be obtained since it is possible to irradiate closely to plant, (3) the most effective monochromatic light in term of spectral sensitivity of pests can be chosen, and (4) light sources of various sizes and shapes can be fabricated.

In Chapter 2, I developed a new experimental tool “LED bottle,” which can provide an efficient experiment. In Chapter 3, effects of light quality and intensity on diapause induction were investigated by utilizing the LED technology. In Chapter 4, the relationship between development and diapause was investigated. In Chapter 5, a spectroscopic approach towards a molecular mechanism of the photoperiodism was conducted.

1.2 Ultraviolet (UV) radiation as a pest control agent

Lubbock (1876) has discovered that insects respond behaviorally to ultraviolet (UV) radiation. Since then, data on the visual sensitivity of insects to UV radiation have been accumulated, indicating that most insects can see UV radiation and utilize it in adjusting their behavior (Silberglied, 1979; Briscoe and Chittka, 1983). Therefore, there is a possibility that UV radiation can be used as an agent, which controls or disturbs behavior of pests.

On the other hand, UV radiation, particularly UV-B radiation (280–320 nm), is harmful to organisms. UV-B radiation is absorbed by certain coenzymes and pigments in vivo, raising these molecules to an excited-state; the excitation energy is finally transferred to H_2O molecules yielding reactive oxygen species (ROS). It is known that the UV-mediated formation of ROS can cause DNA damage (Beehler et al., 1992), and thereby induce lethal oxidative damages particularly on small organisms such as insects and mites.

In Chapter 6, sensitivity to UV radiation in relation to diapause was investigated. In Chapter 7, the production of melatonin known as a ROS scavenger under UV irradiation was investigated.

1.3 Search for phytophagous mite-specific pesticide

Bifenazate [N'-(4-methoxy-biphenyl-3-yl)-hydrazine carboxylic acid isopropyl ester] (IUPAC) is a novel carbazate acaricide discovered by Uniroyal Chemical Company Inc. (now Chemtura Corporation), and this exhibits remarkable properties of specificity being primarily toxic to phytophagous mites including *T. urticae* but non-toxic to predatory phytoseiid mites (Dekeyser et al., 1996). Therefore, such compounds can be used in concurrent chemical and biological control, and contribute to the implementation of IPM. However, the mode of action of bifenazate has not been clarified. This clarification would lead to a screening system for a novel phytophagous mite-specific pesticide. Therefore, I pharmacologically investigated the bifenazate mode of action in Chapter 8.

2 The pest

The order Acarina—mites and ticks—comprises over 20,000 species and includes many important pests of both plants and animals (March, 1976). Spider mites are among the most common plant pests, mainly feeding on leaves, and sometimes causing damage to specific plant parts, e.g., cotyledons, fruits, flowers, fruit spurs or tips of shoots (Tomczyk and Kropczyńska, 1985). The two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is distributed worldwide and is a serious pest affecting a wide variety of crops (van de Vrie et al., 1972).

2.1 Taxonomy

T. urticae belongs to the phylum Arthropoda, subphylum Chelicerata that is separated from insects, the class Arachnoidea where spiders and ticks also belong, and the order Acarina that is separated from spiders. It falls under the genus *Tetranychus* Berlese because the empodium splits distally; usually in 3 pairs of hairs and duplex setae of tarsus I was well separated (Lindquist, 1985).

Koch (1836) gave the first denomination *Tetranychus urticae* in his description in 1836. The mite described by Koch was collected in Germany on the stinging nettle, *Urtica dioica*. It is known that two forms exist in *T. urticae*: green and red forms. Both forms are morphologically very similar and have a worldwide distribution (Carbonnelle and Hance, 2004). However, the green form is generally found in cold and temperature climates, while the red form occurs over much of the warmer temperate zone and subtropics (Dupont, 1979). In the present study, I used the green form of a Japanese population.

2.2 Infestation of *T. urticae* and the damage

Most of spider mites feed underside the leaves and typical symptoms of the feeding are small, light colored punctures which, on prolonged exposure, develop into irregularly shaped, white or grayish-colored spots; changes in color from yellow to bronze are often characteristics of mite attack (Tomczyk and Kropczyńska, 1985).

Low population densities of *T. urticae* on strawberry leaves mainly damage the spongy mesophyll tissue. However, slight injury may be caused to the lowest parenchyma cell layer. Higher densities of spider mite populations on the same plant increased the sphere of damage and more severe injury to palisade parenchyma was then observed (Sances et al., 1979; Kielkiewicz, 1981). In bean leaves inhabited by *T. urticae*, symptoms of injury occurred simultaneously in both spongy and palisade parenchyma layers (Tomczyk and Kropczyńska, 1985).

The thickness of injured leaves may greatly be reduced; a reduction in thickness of approximately 50% in injured bean leaves was reported by Mothes and Seitz (1982). Mite attack decreases the rate of growth in leaf area (Avery, 1962; Avery and Briggs, 1968; Summers and Stocking, 1972), and the number of leaves per plant (Avery, 1962).

2.3 Development and reproduction

In both males and females of *T. urticae*, development proceeds through the following stages: egg, larva, protonymph, deutonymph, and adult. The larval, protonymphal, and deutonymphal stages are further divided into feeding (active) and quiescent (resting) stages. The quiescent stages are referred to as protochrysalis, deutochrysalis, and teleiochrysalis for larval, protonymphal, and deutonymphal stages, respectively. Thus, development of *T. urticae* can be summarized as follows: egg,

larva (including protochrysalis), protonymph (including deutochrysalis), deutonymph (including teleiochrysalis), and adult (Carey and Bradley, 1982; Crooker, 1985).

Adult females normally lay tiny clear spherical eggs of about 0.14 mm in diameter on the underside of leaves where feeding and spinning of delicate webs take place (Davidson and Lyon, 1979; Debach and Rosen, 1991). Eggs may hatch in days to a week into the larval stage (Carey and Bradley, 1982). Larvae are usually colorless with round body and have three pairs of legs. Soon after feeding, their color changes to pale green, brownish green or very dark green and two dark spots appear in the mid-portion of the body (Carey and Bradley, 1982). Towards the end of the feeding stage, the larva attaches itself to the substrate, i.e., leaf, becomes quiescent (protochrysalis), and later molts into a protonymph. The protonymph has four pairs of legs (octapod) and is larger than the larva. Its color is generally pale to dark green and the two spots are larger and more pronounced than in the larva (Carey and Bradley, 1982). Towards the end of the feeding stage, the protonymph attaches itself to the leaf and enters the quiescent stage (deutochrysalis), before molting into a deutonymph. The octapod deutonymph is often larger than the protonymph but both stages share similar color pattern. The deutonymph stage may be significantly longer in the female than in the male (Herbert, 1981a). Females take longer to develop than males, and this may be a general feature of spider mite development (Herbert, 1981a, b). When feeding stops, the deutonymph attaches to the leaf and becomes quiescent (teleiochrysalis). The octapod adult eventually emerges from the teleiochrysalis in as few as five days. At this stage, adult males can be easily distinguished from adult females because of their small size, greater activity, larger legs and more pointed abdomen or wedge-shaped posterior than females (Carey and Bradley, 1982; Crooker, 1985). The mature females are larger than males and show more pigmentation.

Developmental time of *T. urticae* extensively depends on environmental conditions such as temperature, humidity, host plant, leaf age, etc. However, temperature is the most important factor that influences developmental time (Sabelis, 1985a). The lower threshold for development is about 12°C, whereas maximum upper limit to the development is about 40°C (Jeppson et al., 1975). At 20.3°C and relative humidity (RH) of 55% to 98%, the mites developed from eggs to adult in an average of 16.5 days, whereas at 27°C and RH of 90±5%, mites developed from egg to adult in an average of 7.6 days (Shih et al., 1976).

In an optimum condition of approximately 80°F (≈ 27°C), *T. urticae* may complete their development in 5–20 days. Each female mite may deposit nearly 200 eggs and may live for 70 days, thus many overlapping generations can be expected throughout the year. On the average, however, about 70 eggs are deposited and longevity is around 30 days. Unfertilized eggs develop into male mites (Walter and Proctor, 1999).

The shorter time for development particularly contributes high intrinsic rate of natural increase (r_m). Herbert (1981a), working with *T. urticae* on apple, found r_m values ranging from 0.069 per day at 15°C to 0.372 per day at 21°C, at a RH of 80%. Development time was studied and shown to decrease with increasing temperature. Carey and Bradley (1982) have found that the development from egg to adult takes only 6.2 days at 29.4°C, resulting in an r_m of 0.293 per day.

2.4 Diapause

Mites, like insects, show various seasonal adaptations, which enable them to survive periods of unfavorable environmental conditions such as low winter temperatures and periods of heat or drought in the summer (Veerman, 1985). In one of the adaptation, insects and mites have obtained a measure to switch growth, development, or reproduction into either an active or resting state in response to environmental conditions. This arrest is known as diapause. Originally, Wheeler (1893) applied the term "diapause" to the resting stage during embryogenesis, and subsequently its use was altered to refer to suppressed growth, development, or reproduction at any stage of insect development (Andrewartha, 1952; Lees, 1955).

The expression of diapause is controlled by environmental factors via endocrinological shifts (Beck, 1980). Diapause differs from simple quiescence in that quiescence is a direct response to deleterious physical conditions and is terminated as soon as the environmental conditions changed to a favorable one. Diapause, on the other hand, typically begins long before the onset of unfavorable conditions and may not be terminated until long after the disappearance of such conditions (Beck, 1980). The importance of diapause lies not only in ensuring survival through unfavorable seasons, but also in the regulation of seasonal phonologies by synchronization of life cycles and the determination of patterns of voltinism (Danilevskii, 1965; Tauber and Tauber, 1978; Tauber et al., 1986).

In *T. urticae*, only adult females enter diapause to overwinter by sensing long-night conditions, and reproduction is inhibited in diapause females (Veerman, 1985). However, until the early 1950s it was generally believed that the condition of the host plant was the major factor determining diapause in spider mites (e.g. Pritchard

and Baker, 1952). The role of photoperiod in the induction of diapause was first demonstrated by Lees (1950) and Miller (1950) for the fruit tree red spider mite (European red mite) *Panonychus ulmi* and by Bondarenko (1950) for *T. urticae*.

Diapausing females are bright orange (Fig. 1) and do not feed (Veerman, 1985). Oxygen consumption in diapausing females was shown to be very low compared to that of active (non-diapausing) females (McEnroe, 1961; Geispitz and Orlovskaja, 1971).

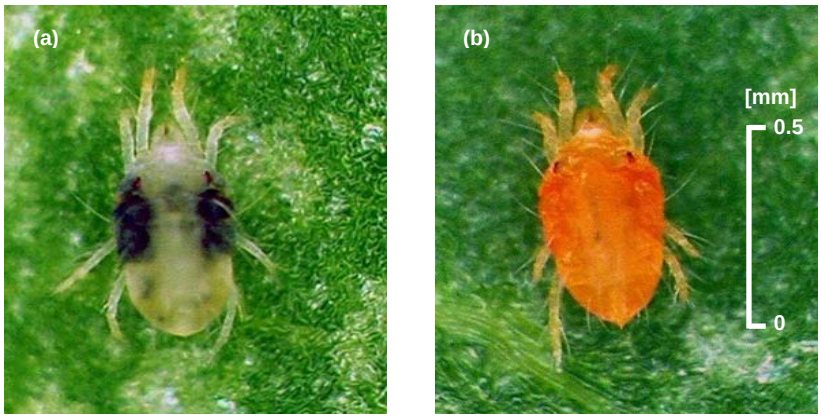

Fig. 1 Photographs of (a) non-diapausing (reproductive form) and (b) diapausing (overwintering form) females of *Tetranychus urticae*. The non-diapausing female is yellowish-green and has two dark spots. The diapausing female is bright orange and has no dark spots.

Resistance to cold is stronger in diapausing females than in non-diapausing females (Veerman, 1985). Diapause females overwintering outdoors have been reported to withstand minimum temperatures of −13°C (Lloyd, 1922) and −27°C (Bondarenko, 1958); in laboratory tests van de Bund and Helle (1960) and Geispitz and Orlovskaja (1971) showed that diapausing females could survive temperature of −22°C and −24°C respectively. However, no mites survived under −32°C (Bondarenko, 1958). According to Helle (1962), diapausing females could be preserved for at least 8 months at –2°C, on condition that they were protected from drying. At 6°C, many mites held at RHs of 75% and 93% survived for more than 8 months, nearly twice as

long as those kept at RH of only 40% (Parr and Hussey, 1966). Stenseth (1965) showed that survival of diapausing females was much higher at a constant temperature of −15°C than that of non-diapausing females. While freezing is fatal to the mites, they can survive temperatures below 0°C in a supercooled state. The supercooling point was shown to be −18.7°C for diapausing females and −22.4°C in non-diapausing females; no correlation was found between the supercooling points of the different forms and their survival at constant low temperature (Stenseth, 1965). Different results were reported by Geispitz et al. (1971) who found higher supercooling points in non-diapausing females (in the range −12.6 to −16.9°C) than in diapausing females (−18.3 to −24.4°C) of a Russian strain of *T. urticae*.

Diapausing females have a positive geotaxis (Foott, 1965) and negative phototaxis (Bondarenko, 1958; Hussey and Parr, 1963; Parr and Hussey, 1966; McEnroe, 1971) and migrate from the host plants. In greenhouses, the females have been found overwintering in cracks and crevices in the house structure, supporting stakes, hollow stems and straws on the beds, irrigation equipment, door locks, and pipe fittings (Hussey, 1972; French and Ludlam, 1973). In open field, overwintering females have been found, often in great numbers, in clods of soil in apple orchards (Weldon, 1910), hop gardens (Massee, 1942), blackcurrant plantations (Collingwood, 1955), and in clay soils (Helle, 1962). However, it appeared that in most cases mortality of mites overwintering in the soil was high (Veerman, 1985). Overwintering females have also been found in the cracks of trees, under the bark of poles (Massee, 1942; Helle, 1962), on dried leaves (Massee, 1942) and straw (Collingwood, 1955), and in hollow withered flower stems (Helle, 1962). On woody host plants, overwintering females have been found under the bark (Helle, 1962; Uchida, 1980).

Chapter 2

Effects of extending the light period on diapause induction in a Japanese population of the two-spotted spider mite, *Tetranychus urticae*

Abstract

Artificial lighting is a merit of a "plant factory," which might be utilized to suppress an increase in pest population. I investigated the effects of extending the light period on diapause induction in the two-spotted spider mite, *Tetranychus urticae*. The mites were reared at 18°C under light periods ranging from 2 to 64 h combined with a constant dark period of 16 h in aluminum bottles, with white light emitting diodes attached inside to minimize fluctuations in air temperature between the light and dark periods. Diapause was induced in adult females when the light period was 24 h or shorter, and diapause induction was inhibited when the light period extended over 32 h. The development of deutonymphs was delayed under a diapause-inducing photoperiod. Diapause inducing photoperiods may suppress an increase in the mite population, by slowing down development and reproduction.

Introduction

Implementation of integrated pest management (IPM) by using low amounts of pesticides is an urgent need. Although IPM employs all possible measures to control pests, physical measures are under-exploited, though equally effective to biological measures. One effective application of physical measures is the manipulation of lighting. I here attempted to control the life cycle of the two-spotted spider mite by photoperiodic manipulation.

Diapause is an adaptation to cope with unfavorable seasons (Tauber et al., 1986), but if it is untimely induced, it hinders population growth. Cumulative data have shown that in long-day type pests, diapause is prevented by (1) light breaks adequately placed at night, and (2) extension of the light period (Ankersmit, 1968; Beach and Craig, 1979; Berlinger and Ankersmit, 1976; Hayes et al., 1970, 1974a, b, 1979; Sáringer, 1982, 1983; Sullivan et al., 1970). Unfortunately, such measures have not been developed further since it is economically difficult to obtain sufficient light intensity in the field.

It is easy to control the light environment in “plant factories”, such as the closed plant production systems using artificial light only (Kozai and Ohyama, 2006), where a photoperiod that induces diapause in the pest can easily be installed. The closed plant production system has been increasingly used commercially in Japan and Korea for the production of tomato, cucumber and eggplant transplants, and leafy vegetables (Kozai and Ohyama, 2006). With this system, in addition to photoperiod, light intensity and quality, air temperature, humidity, CO_2 concentration and air current speed can be controlled as desired.

Tetranychus urticae is distributed worldwide and is a serious pest for a wide variety of crops (van de Vrie et al., 1972). Only adult females enter diapause, and reproduction is inhibited in diapause females (Veerman, 1985). In a 24 h light-dark cycle, females that experienced a short day during the developmental stages (larval and nymphal stages) entered diapause at the adult stage (Parr and Hussey, 1966; Veerman, 1985). Veerman (2001) suggested that the photoperiodic time is measured in insects and mites by a non-circadian "hourglass" that determines the qualitative difference between "long" and "short" dark periods, i.e., longer or shorter than a critical night length (CNL), and diapause is induced under a dark period that lasts longer than a CNL. However, since diapause is not induced under continuous darkness (Veerman, 1977), a certain light period is necessary for diapause induction. Also, the light period is important environmental factor in plant growth (e.g. Garner and Allard, 1927).

This study investigates the effects of extending the light period on diapause induction in a Japanese population of *T. urticae* to assess the range of photoperiods that support plant growth but discourage an increase in the mite population.

Materials and Methods

Laboratory culture of T. urticae

The founder mite population was collected from an apple orchard (*Malus pumila* Mill. cv. Fuji) at Iwate, Japan (39°41′N) in 2001. The offspring populations were maintained in the laboratory on leaves of kidney bean (*Phaseolus vulgaris* L.) under long-day (LD 16:8) condition by using white fluorescent lamps, at 25°C.

Thirty adult females were introduced onto a fresh bean leaf that was placed on water-soaked cotton in a plastic petri dish (9 cm diameter, 2 cm deep), and they were

allowed to lay eggs for 12 h under continuous darkness at 25°C. The eggs were maintained for 5 days under the same environmental conditions.

Experimental device

Aluminum "photoperiodic bottles" (300 ml; Fig. 2.1) were used, placed in an incubator (MIR-553; SANYO Electric Co. Ltd., Osaka, Japan). Each bottle had 16 kidney bean leaf disks (10 mm diameter) placed on water-soaked cotton and was equipped with a single white light emitting diode (LED) (NSPW500BS; Nichia Co., Tokushima, Japan) that was wired to externally mounted time switches (TB23; Matsushita Electric Industrial Co. Ltd.). Air exchange was provided by four 10-mm holes per bottle, covered with a gas-permeable membrane filter (Milliseal; pore diameter, 0.5 μm, Nihon Millipore K.K., Tokyo, Japan).

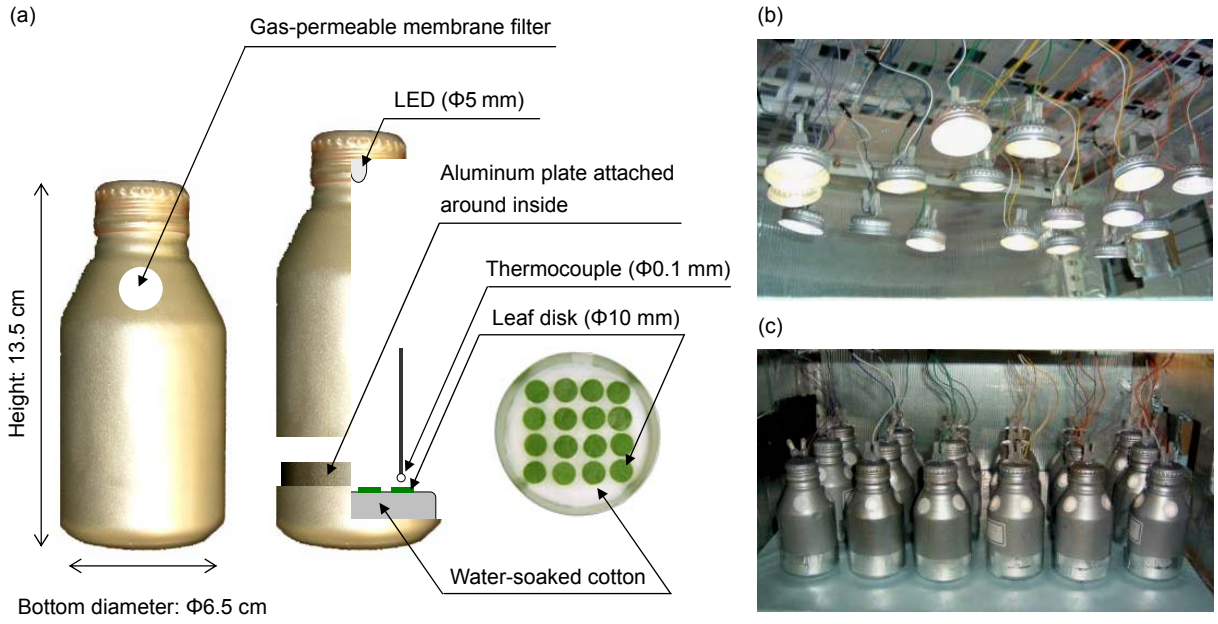

Fig. 2.1 (a) Schematic diagram of an LED bottle (sectional side and top views). The bottle has one section at a height of 2 cm above the bottom for setting leaf disks. (b) White LEDs (NSPW500BS; Nichia Co., Tokushima, Japan) attached to the underside of the bottle caps. (c) Photoperiodic bottles in an incubator.

Experimental conditions

Larvae that hatched 5 days after egg laying were transferred onto the leaf disks (1 mite/disk). The light periods were set at 2-64 h (see Table 2.1). In all treatments, the dark period was fixed at 16 h, which was longer than CNL. Light intensity at the bottom of the bottle was set at 3 W m^{-2} and air temperature was set at 18.0°C at a height of 1 mm above the leaf disk. Air temperature was measured with a copper-constantan thermocouple (0.1 mm wire diameter). Every 2 min, measurements were recorded using a data logger (GK100; ESD Co. Ltd., Tokyo, Japan) and stored in a computer.

Observation

By using a stereomicroscope (SZ-PT; Olympus Co., Tokyo, Japan) to determine the developmental times of the larval, protonymphal, and deutonymphal stages, individual females were observed every 24 h until they reached the adult stage. At the adult stage, sex was judged and only female records were used for determination of developmental times. Under light-dark cycles other than the 24 h one, females were often observed in the dark period by using a lighting system (red LEDs) that the mite cannot sense; thus, unwanted light breaks were prevented. In a preliminary experiment, I investigated the sensitivity of the photoreceptor associated with diapause induction to light quality. Exposure of juvenile mites to red LED (GL5UR3K1; SHARP Co., Osaka, Japan) light did not induce diapause (0%) even under the short-day (LD 8:16) condition (Suzuki et al., 2008a). Figure 2.2 shows the spectral distribution of the white and red LEDs. Mites could not sense the red light and experienced the photoperiod as continuous darkness. This is comparable to results obtained with light filters conducted with *Panonychus ulmi* (Lees, 1953) and *T. urticae* (Veerman and

Veenendaal, 2003).

Diapause induction was judged 7 days after adult emergence. Adult females were considered to be in diapause if their body color had changed from yellowish-green to uniform deep orange-red due to the accumulation of keto-carotenoids (Veerman, 1974). The leaf disks were renewed every 5 days.

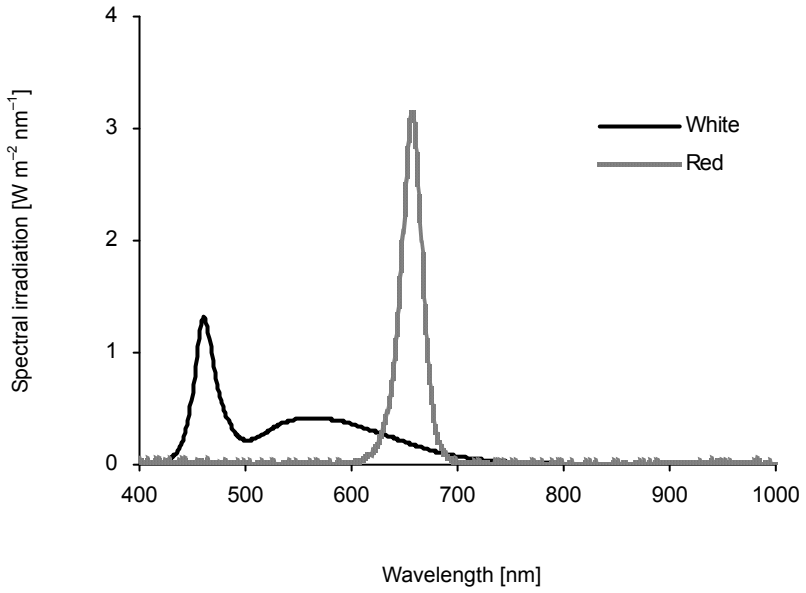

Fig. 2.2 Spectral distributions of white (NSPW500BS; Nichia Co., Tokushima, Japan) and red LEDs (GL5UR3K1; SHARP Co., Osaka, Japan) when the irradiance was set at 100 W m^{-2} (400–1000 nm).

Results

Air temperature

In all treatments, the average temperature at 1 mm above the leaf disk were similar during the light and dark periods (Table 1), because a single LED generates little heat and aluminum has a high thermal conductivity (237 W m^{-1} K^{-1}).

Table 2.1 Air temperatures (mean ± SD) in the bottle at a height of 1 mm above the leaf disk during the light and dark periods.

Treatment [h]		Air temperature [°C]	
Light period	Dark period	Light period	Dark period
2	16	18.1 ± 0.1	18.0 ± 0.1
4	16	18.1 ± 0.2	18.0 ± 0.1
8	16	17.9 ± 0.2	17.8 ± 0.1
16	16	17.9 ± 0.1	17.8 ± 0.1
24	16	17.9 ± 0.1	17.9 ± 0.1
32	16	18.0 ± 0.1	17.9 ± 0.1
40	16	18.0 ± 0.1	18.0 ± 0.1
48	16	18.0 ± 0.1	18.0 ± 0.1
56	16	18.1 ± 0.2	18.0 ± 0.1
64	16	18.0 ± 0.2	17.9± 0.1

Diapause induction

Diapause percentage under the 2 h light period exceeded 80%, and it almost reached 100% under the 4 to 24 h light periods (Fig. 2.3). Under the 32 h light period, the diapause percentage decreased to 70%, which was significantly lower than that observed under the 4 to 24 h light periods. At longer light periods diapause percentage decreased further, until no diapause females were observed under the 56 and 64 h light periods.

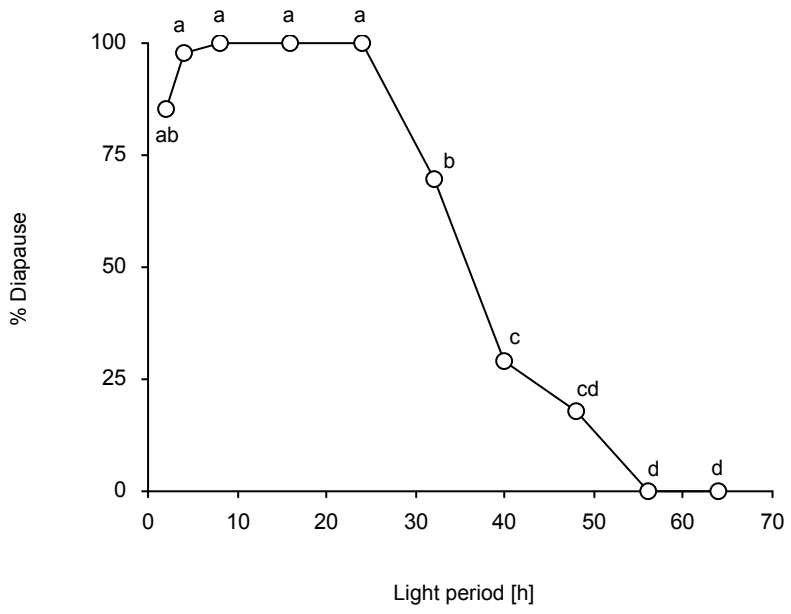

Fig. 2.3 Effects of extending the light period on the percentage of diapause induction in *Tetranychus urticae*. Dark period was a constant of 16 h. Different letters indicate a significant difference at $p < 0.05/45$ by Fisher's exact test with Bonferroni correction. Number of females under light period of 2, 4, 8, 16, 32, 40, 48, 56, and 64 h is 34, 45, 25, 25, 38, 56, 45, 34, 31, and 28, respectively.

Developmental time

The developmental times at the larval and protonymphal stages were slightly shorter when the light period was longer, but the differences were not significant (Table 2.2). Interestingly, significant correlations ($P < 0.05$, Scheffe post-hoc test) were found between light period and the deutonymphal stage and the total developmental time: the shorter the light period, the longer the deutonymphal stage and the longer the total developmental time.

Table 2.2 Developmental times (mean ± SE) of the females of *Tetranychus urticae* at each immature stage under different light periods with a constant dark period of 16 h.

Light period [h]	*N*	Larva [d]	Protonymph [d]	Deutonymph [d]	Total [d]
2	34	5.2 ± 0.5a*	4.9 ± 0.1a	6.5 ± 0.1ab	16.7 ± 0.5a
4	45	5.0 ± 0.3a	4.8 ± 0.1a	6.6 ± 0.1a	16.4 ± 0.3a
8	25	5.5 ± 0.3a	4.7 ± 0.1a	5.9 ± 0.2abcd	16.1 ± 0.4ab
16	25	5.0 ± 0.2a	4.8 ± 0.1a	6.1 ± 0.1abc	15.9 ± 0.3ab
24	38	5.1 ± 0.3a	5.0 ± 0.2a	6.5 ± 0.1ab	16.6 ± 0.4a
32	56	4.7 ± 0.2a	4.5 ± 0.1a	5.7 ± 0.1bcde	14.9 ± 0.2abc
40	45	4.4 ± 0.2a	4.5 ± 0.1a	5.2 ± 0.1cde	14.1 ± 0.3bc
48	34	4.0 ± 0.2a	4.6 ± 0.1a	5.1 ± 0.2cde	13.8 ± 0.4bc
56	31	4.3 ± 0.2a	4.4 ± 0.2a	4.8 ± 0.1de	13.5 ± 0.3c
64	28	4.6 ± 0.2a	4.3 ± 0.1a	4.6 ± 0.2e	13.5 ± 0.3c

*Values in the same column followed by different letters are significantly different at $P < 0.05$ as determined by the Scheffe post-hoc test after the Kruskal-Wallis test.

Comparison of the developmental times between diapause and non-diapause females under light periods of 32, 40, and 48 h shows a significant difference only at the deutonymphal stage; this lasts longer in females that enter diapause when adult (Fig. 2.4).

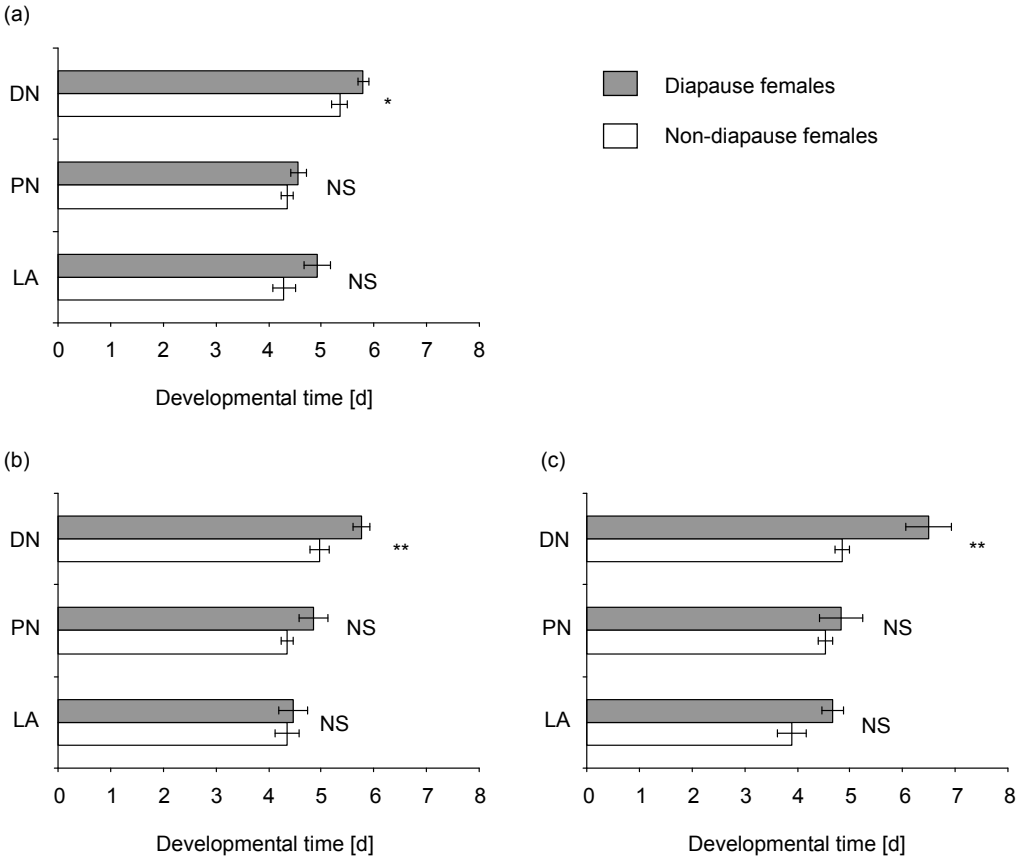

Fig. 2.4 Comparison of the developmental times between diapause and non-diapause females of *Tetranychus urticae* under light periods of (a) 32, (b) 40, and (c) 48 h. Dark period was a constant of 16 h. LA, PN, and DN indicate the larval, protonymphal, and deutonymphal periods, respectively. Horizontal bar indicates SE of the mean. NS: non-significant; *, **: significant at $P < 0.05$ and 0.01, respectively, by Mann-Whitney *U*-test. Number of diapause and non-diapause females is (a) 17 and 39, (b) 32 and 13, and (c) 28 and 6, respectively.

Discussion

To investigate the light environment that induces diapause of the mite population, I developed a new experimental tool, i.e., the "photoperiodic bottle." The light from the LEDs rarely leaks out of the bottle; therefore, various photoperiods could be formed within a single incubator.

The bottle minimizes the fluctuations in air temperature between the light and dark periods. This is important because even small temperature differences can profoundly affect the expression of diapause, as was for example shown by Lees (1953) for *P. ulmi* and Veerman (1977) for *T. urticae*. Buffering a potential effect of thermoperiod allowed to focus on the effects of photoperiod on diapause induction. The photoperiodic bottle is effective and handy when a photoperiodic experiment is conducted with small organisms.

Vaz Nunes and Veerman (1986) performed a resonance experiment with a Dutch population of *T. urticae*, in which a constant dark period was combined with variable light periods. No sign of rhythmicity was observed in the response curves under a constant dark period of 12 h; high diapause percentage was observed with a light period varying from 5 h to approximately 32 h. With even longer light periods, diapause percentage decreased gradually and reached 10% for a light period of 60 h. This result is comparable to mine with the Japanese population of *T. urticae* and a 16 h dark period.

Saunders (1982) introduced the concept of a "photoperiodic counter" system wherein the number of long-night cycles should exceed a critical number in order to induce diapause in most insects and mites. Accordingly, photoperiodism in insects and mites is considered to comprise not only the photoperiodic clock but also the counter system that accumulates the photoperiodic information contained in a sequence of light-dark cycles. Veerman and Vaz Nunes (1987) reported that diapause percentage in

T. urticae increased as the number of long-night cycles increased, suggesting the existence of a photoperiodic counter system. Therefore, in my experiment the inhibition of diapause induction under extended light period in a non-24 h-photoperiod might be due to the accumulation of too few light-dark cycles.

Post-embryonic development was retarded as the light period decreased. Moreover, the deutonymphal period of diapause females was significantly longer than of non-diapause females. This is the first report on the association between photoperiodism and immature development in *T. urticae*.

In conclusion, the present study demonstrated that diapause and the delay of development in the mites were induced by providing photoperiods which the light period was 24 h or shorter with 16 h dark period. Further studies to analyze the effect of such photoperiods on plant growth are required, to optimize the effectiveness of pest suppression by means of physical environment manipulation in closed plant production systems.

Chapter 3

Effects of light quality and intensity on diapause induction in the two-spotted spider mite, *Tetranychus urticae*

Abstract

It has been known that diapause is not induced in populations of the two-spotted spider mite, *Tetranychus urticae*, under continuous darkness (DD). When the light intensity was changed under LD 8:16 at 18°C, the threshold intensity for blue light (475 nm) to induce 50% of diapause was below 50 mW m^{-2}; and the blue light was most effective in photoperiodic induction of diapause. The threshold intensities for green (572 nm) and orange (612 nm) light were 50–500 and 500–2500 mW m^{-2}, respectively. *T. urticae* showed no sensitivity to red light (658 nm) even at 2500 mW m^{-2} intensity. Low diapause incidence under long wavelengths was probably due to the absence of photoreception by photoreceptor pigments for photoperiodic time measurement, or for circadian entrainment required for photoperiodic induction.

Introduction

The two-spotted spider mite, *Tetranychus urticae* is distributed worldwide and is a serious pest for a wide variety of crops (van de Vrie et al., 1972) as a typical *r*-strategist (Sabelis, 1985b). The *r* is elevated by a short developmental period and high fecundity enforced by life-history characteristics to produce successive generations. Growth rate and photoperiodism are important determinants in life history.

Veerman and his colleagues performed phenomenological investigations for the photoperiodic mechanism of diapause in *T. urticae*. They proposed a photoperiodic model based on three characteristics, namely, (1) a non-repetitive long-night measuring photoperiodic timer, which may be either a real hourglass or a rapidly damped oscillator; (2) since Nanda-Hamner protocol produced rhythmic fluctuations in diapause induction, circadian system is somehow involved in the photoperiodic response, and (3) the so-called photoperiodic counter, which integrates photoperiodic "information" (Vaz Nunes and Veerman, 1982, 1986; Veerman and Vaz Nunes, 1987; Veerman, 2001; Veerman and Veenendaal, 2003). However, the basic knowledge on several aspects, particularly, hormonal mechanism of diapause regulation, molecular mechanism of circadian system and its involvement in photoperiodism, and photoreception mechanism still remain unclarified.

The present study investigates the sensitivity of *T. urticae* to quality and intensity of light for photoperiodic induction of diapause by using light emitting diodes (LEDs).

Materials and Methods

Laboratory culture of T. urticae *and the food plant*

The founder population of *T. urticae* was collected from an apple orchard (*Malus pumila* Mill. cv. Fuji) at Iwate, Japan (39°41′N), in 2001. The offspring populations were maintained in the laboratory on leaves of kidney bean (*Phaseolus vulgaris* L.) under long-day (LD 16:8) conditions provided by white fluorescent lamps (FPL27EX-N; Matsushita Electric Industrial Co. Ltd., Osaka, Japan) at 25°C.

Thirty adult females were introduced onto a fresh bean leaf that was placed on water-soaked cotton in a plastic petri dish (9 cm diameter, 2 cm depth), and they were maintained at 25°C for 12 h in continuous darkness (DD) for oviposition. The eggs were maintained for 5 days under the same environmental conditions.

The kidney beans were planted in plastic pots (7.5 cm diameter, 6.5 cm depth) containing moist soil (Yanmar Agricultural Equipment Co. Ltd., Osaka, Japan) and cultivated in a closed transplant production system at Chiba University (Chun and Kozai, 2000) under a controlled environment. The environmental conditions inside the system were LD 16:8 under white fluorescent lamps (FHF32EX-N-H; Matsushita Electric Industrial Co. Ltd.), PPFD (photosynthetic photon flux density) of 300 μmol m^{-2} s^{-1}, air temperature of 25°C, 70% RH, CO_2 concentration of 1500 μmol mol^{-1} and air current speed of 1.3 m s^{-1}. Half-strength Otsuka A prescription nutrient solution (Otsuka Chemical Co. Ltd., Osaka, Japan; pH, 6.0; EC, 1.2 dS m^{-1}) was used to irrigate the plants every day.

Experimental devices

Experimental devices were made according to Suzuki et al. (2007) with some modifications. All experiments were conducted using incubators (MIR-152; SANYO Electric Co. Ltd., Osaka, Japan) equipped with either blue (E1L51-3B; Toyoda Gosei Co. Ltd., Aichi, Japan), green (TLGE183P; Toshiba Co., Tokyo, Japan), orange (TLOE180AP; Toshiba Co.), red (GL5UR3K1; SHARP Co., Osaka, Japan) or white (NSPW500BS; Nichia Co., Tokushima, Japan) LEDs. Each LED unit was wired to an externally mounted DC power supply (PR18-5A; Kenwood Co., Tokyo, Japan) with a time switch (TB23; Matsushita Electric Industrial Co., Ltd.). Figure 3.1 shows the spectral distribution of LEDs. The light intensity was regulated by electric current from the DC power supply. A rearing system consists of 36 kidney-bean leaf disks (10 mm diameter) placed on water-soaked cotton in a petri dish and mites were placed on the disks.

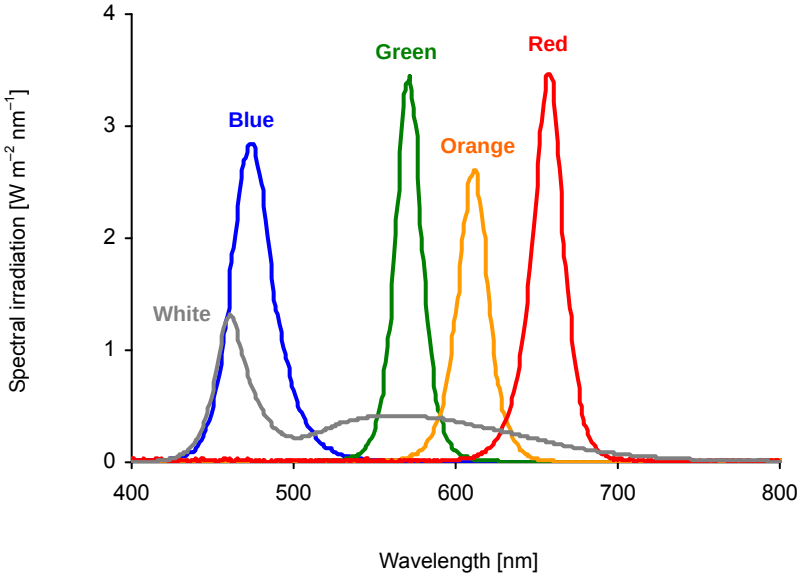

Fig. 3.1 Spectral distributions of blue (E1L51-3B; Toyoda Gosei Co. Ltd., Aichi, Japan), green (TLGE183P; Toshiba Co., Tokyo, Japan), orange (TLOE180AP; Toshiba Co.), red (GL5UR3K1; SHARP Co., Osaka, Japan) and white LEDs (NSPW500BS; Nichia Co., Tokushima, Japan) when the irradiance was set at 100 W m^{-2} (400-800 nm).

Experimental conditions

Larvae that hatched 5 days after oviposition were transferred onto the leaf disks (1 mite/disk). Under blue, green and orange light, the intensity was set at 50, 100 and 2500 mW m^{-2} on the petri dish. Under red and white light, the intensity was set at 2500 mW m^{-2} on the petri dish. In all treatments, the light and dark periods were set at 8 and 16 h, respectively (LD 8:16). Air temperature was set at 18.0°C at a level of 1 mm above the leaf disk. Air temperatures were measured with copper-constantan thermocouples (wire diameter 0.1 mm). The measurements were recorded using a data logger (CR23X; Campbell Scientific Inc., Utah, USA) and stored in a personal computer.

Observation

Diapause induction was judged 11 days after adult emergence by the lack of oviposition (Veerman, 1985). The leaf disks were renewed every 5 days.

Results

Table 3.1 shows the diapause incidence under LD 8:16 consisting of white or red (658 nm) light and darkness, or in DD. Under white light at 2500 mW m^{-2}, *T. urticae* recognized the photoregimen as a long night of 16 h, longer than the critical night length under which all females entered diapause. On the other hand, none of them entered diapause under red-light conditions even if the intensity was as high as 2500 mW m^{-2} and in DD. *T. urticae* recognized the red light as darkness, and the LD cycle was regarded as DD.

Table 3.1 Effect of light quality on diapause induction in *Tetranychus urticae* under LD 8:16 at 18°C.

Light source [Wavelength peak (nm)]	Irradiance [mW m^{-2}]	*N*[a]	% Diapause
White LED	2500	25	100
Red LED [658]	2500	16	0
None[b]	–	75	0

[a]Number of individuals. [b]Continuous darkness.

When *T. urticae* was reared under different light qualities at identical light intensities, namely, at 50 and 500 mW m^{-2}, diapause incidence decreased as the wavelength increased, and the differences among the light qualities were significant (Fig. 3.2a, b; $P < 0.05/3$, Fisher's exact test with Bonferroni correction). However, at 2500 mW m^{-2}, *T. urticae* recognized this as light (therefore as a long night) and nearly all females subsequently entered diapause regardless of the light qualities. At this intensity, the differences among the light qualities were not significant (Fig. 3.2c, $P > 0.05/3$).

A comparison of the diapause incidences at different light intensities under identical light qualities suggested that *T. urticae* recognized the photoperiod of blue light (475 nm) as a long night and nearly all females entered diapause even if the light intensity was as low as 50 mW m^{-2}. Differences among the light intensities were not significant (Fig. 3.2d, $P > 0.05/3$). Under green-light conditions (572 nm), diapause incidence increased with the light intensity and the differences were significant (Fig. 3.2e, $P < 0.05/3$). Under orange-light conditions (612 nm), diapause females appeared only at 2500 mW m^{-2}. However, *T. urticae* could not recognize this photoperiod regimen as a long night at intensities below 500 mW m^{-2} where diapause incidences were significantly lower than at 2500 mW m^{-2} (Fig. 3.2f, $P < 0.01/3$). The data suggest that the threshold intensity under blue-light conditions for 50% diapause induction was below 50 mW m^{-2} and threshold intensities under green- and orange-light conditions were 50–500 and 500–2500 mW m^{-2}, respectively.

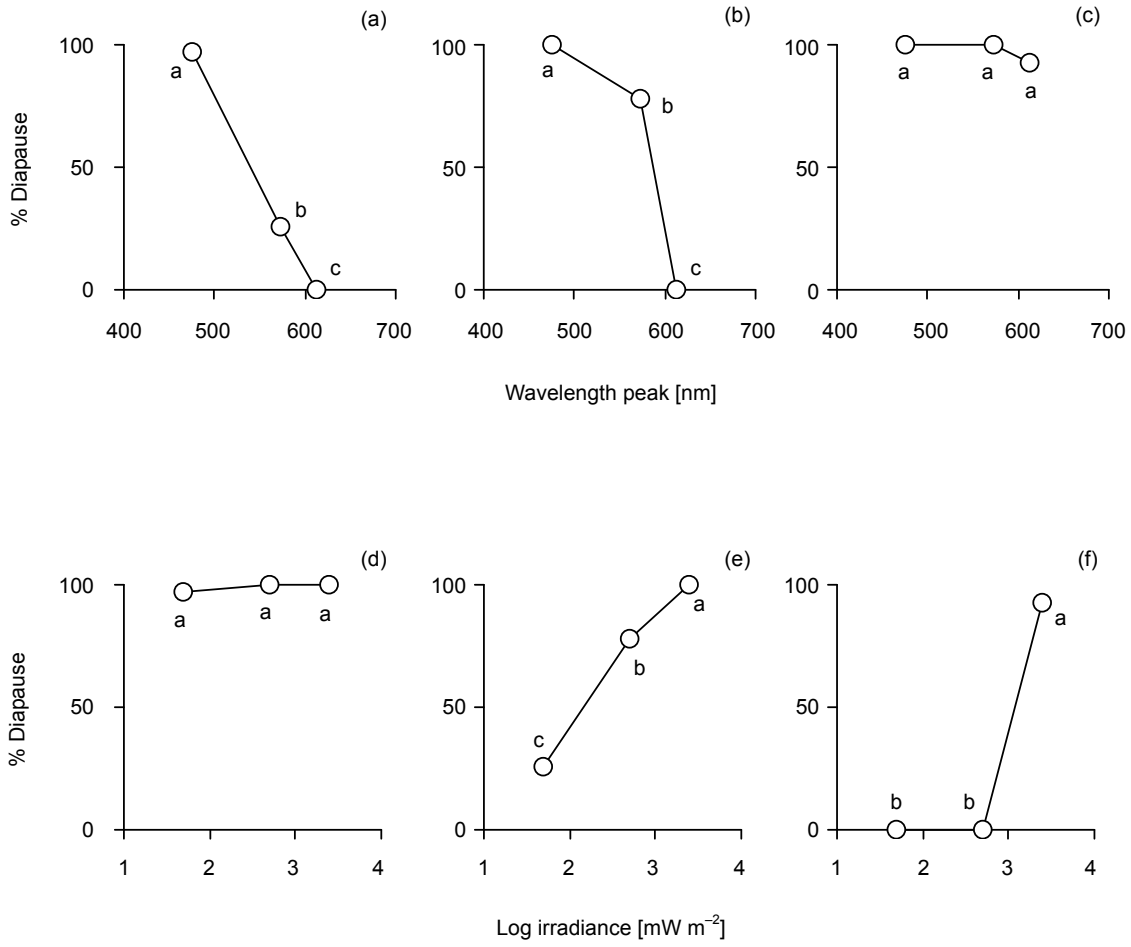

Fig. 3.2 A comparison of diapause incidence in *Tetranychus urticae* under LD 8:16 at 18°C between light quality under each light intensity (a: 50 mW m^{-2}, b: 500 mW m^{-2}, c: 2500 mW m^{-2}) and between light intensity under each light quality (d: blue, e: green, f: orange). Different letters indicate significant differences at $P < 0.05/3$ by Fisher's exact test with Bonferroni correction, $N = 28–42$

Discussion

The results of the present study revealed that in the range of visible-light wavelengths, blue light (475 nm) was the most effective for photoperiodic induction of diapause in *T. urticae* and red light (658 nm) had no effect on photoperiodism.

Veerman (2001) has suggested that the photoperiodic clock in insects and mites most probably operates as a non-circadian hourglass with an opsin-based photoreceptor that is coupled with a carotenoid-derived chromophore, and that the circadian system plays certain roles in insect and mite photoperiodism, as shown in the case of the Nanda-Hamner rhythm (controlled by a different photoreceptor, possibly cryptochrome).

The evidence that the photoreceptor pigment for photoperiodism is a carotenoid-derived chromophore coupled with an opsin originated from diet manipulation (Veerman and Helle, 1978; Veerman, 1980; Van Zon et al., 1981; Veerman et al., 1983; Shimizu and Kato, 1984; Veerman et al., 1985; Hasegawa and Shimizu, 1988; Bosse and Veerman, 1996). On the other hand, the photoreceptor pigment for circadian entrainment might well be a vitamin B_2-based cryptochrome, as demonstrated for *Drosophila* (for overview, see Hall, 2000; Van Gelder, 2002).

To conclude that different photoreceptor pigments are involved in circadian entrainment and in photoperiodism, Veerman and Veenendaal (2003) conducted the following experiment with *T. urticae*. The *T. urticae* population used in their study appeared to be insensitive to red light (>600 nm), but strong photoperiodic sensitivity was found to orange-red light (>580 nm): *T. urticae* discriminated a long night of 12 h from a short night of 8 h of orange-red light at 720 mW m^{-2}, by producing high and low diapause incidence, respectively, but only in regimens with an LD cycle (*T*) of 20 h;

surprisingly, the "normal" long night regimen of LD 12:12 with T = 24 h virtually resulted in no diapause induction under orange-red light. In *T. urticae*, the Nanda-Hamner rhythm (which had a period of the free-running rhythm, the so-called τ value) is only 20 h, and desynchronization of τ with T disturbed the photoperiodic induction of diapause (Veerman and Vaz Nunes, 1980). Therefore, they suggested that the virtual absence of diapause in orange-red light would result from rapid desynchronization of the Nanda-Hamner rhythm under LD cycles having T = 24 h but not in T = 20 h. In white light at 720 mW m^{-2} and even at 180 mW m^{-2}, the Nanda-Hamner rhythm was entrained to both T = 20 h and T = 24 h, and diapause was induced in both long-night regimens (LD 8:12 and 12:12). Therefore, they suggested that in *T. urticae*, different pigments are involved in photoperiodism and in the entrainment of the circadian rhythm, i.e., the Nanda-Hamner rhythm; if only one pigment was involved, both long-night regimens (LD 8:12 and 12:12) would have produced the same results in orange-red light (either diapause or non-diapause), as in the case of white light.

The present study showed that no diapause induction occurred in red light (658 nm), as in DD (Table 3.1). The threshold intensity in red light for 50% diapause induction could be above 2500 mW m^{-2}; the photoreceptor pigment for photoperiodism and/or circadian system in *T. urticae* had low or no sensitivity to red light. Therefore, *T. urticae* could not recognize the long-night cycles (LD 8:16) by the photoreceptor pigment for photoperiodism, or desynchronization of the Nanda-Hamner rhythm having τ = 20 h from the LD cycle (T = 24 h) occurred. This sensitivity to red light is comparable with those of the action spectra for photoperiodic responses in *Bombyx mori* (Kogure, 1933), *Metatetranychus ulmi* (=*Panonychus ulmi*) (Lees, 1953), *Antheraea pernyi* (Hayes, 1971), *Megoura viciae* (Lees, 1971, 1981; Hardie et al., 1981), and

Aleyrodes proletella (Adams, 1986). Using *Pectinophora gossypiella*, Pittendrigh and Minis (1971) found that the eclosion rhythm and oviposition rhythm were entrained by blue light (480 nm) but not by red light (600 nm). However, photoperiodic induction of diapause could be controlled successfully by using wavelengths of 600 nm and above. This suggests that in *P. gossypiella*, different photoreceptor pigments are involved in circadian entrainment and in photoperiodism. Further, among insects and mites, there exists a photoreceptor pigment for photoperiodism, which may be coupled with a carotenoid-derived chromophore but shows different sensitivity to light quality, e.g., short- and long-wave opsin.

Low diapause incidence was observed under orange-red light (>580 nm) at 720 mW m^{-2} under an LD cycle with T = 24 h (Veerman and Veenendaal, 2003), whereas strong photoperiodic sensitivity was found in orange light (612 nm) at 2500 mW m^{-2} (Fig. 3.2f). This suggests that the Nanda-Hamner rhythm in *T. urticae* was entrained by the LD cycle with T = 24 h when the intensity of orange light was as high as 2500 mW m^{-2}, i.e., not only the photoreceptor pigment for photoperiodism but also that for circadian entrainment in *T. urticae* shows a slight sensitivity to orange light.

A high sensitivity was observed in the case of blue light (Fig. 3.2d); additionally, the photoreceptor pigment for photoperiodism could recognize the long-night cycles (LD 8:16), and the Nanda-Hamner rhythm was entrained by the LD cycle with T = 24 h even if the intensity of blue light was as low as 50 mW m^{-2}. This high sensitivity to blue light is also comparable with those of the photoperiodic responses in *B. mori* (Kogure, 1933), *M. ulmi* (Lees, 1953), *A. pernyi* (Hayes, 1971), *M. viciae* (Lees, 1971, 1981; Hardie et al., 1981), *A. proletella* (Adams, 1986), and with those of the circadian responses in *Drosophila* (Frank and Zimmerman, 1969) and *P. gossypiella* (Bruce and Minis, 1969; Pittendrigh and Minis, 1971). This suggests that in most insects and

mites, the photoreceptor pigment for photoperiodism has a high sensitivity to blue light and that the photoreceptor pigment for circadian entrainment is also a blue-light sensitive photoreceptor such as cryptochrome.

Chapter 4

Diapause-inducing stimuli inhibit nymphal development in the two-spotted spider mite, *Tetranychus urticae*

Abstract

Females of the two-spotted spider mite (*Tetranychus urticae*) were grown under different photoperiods, and the photoperiodic regulation of diapause was examined. The photoperiodic-response curve for diapause incidence was of the long day–short day type having critical day lengths (CDLs) of 2 and 12.5 h; diapause was induced between these CDLs. The preimaginal period in diapausing females was significantly longer than in non-diapausing females; moreover, a significant positive correlation was detected between diapause incidence and developmental time in the deutonymphal stage. Diapause incidence was high when long-night cycles were applied against a background of continuous darkness in the deutonymphal stage. These observations suggest that diapause-inducing conditions inhibit nymphal development, particularly, in the deutonymphal stage when photoperiodic time measurement for determination of reproduction or diapause was carried out.

Introduction

Tetranychus urticae is distributed worldwide and is a serious pest affecting a wide variety of crops (van de Vrie et al., 1972). Only the adult females of *T. urticae* enter diapause and reproduction is inhibited in diapausing females (Veerman, 1985).

Veerman and his colleagues performed phenomenological investigations for the photoperiodic mechanism of diapause in *T. urticae*. They constructed a photoperiodic model based on three characteristics (1) a non-repetitive long-night measuring photoperiodic clock, which may be either an hourglass or a rapidly damped oscillator; (2) since Nanda-Hamner protocol produced rhythmic fluctuations in diapause induction, circadian system is somehow involved in the photoperiodic response, and (3) the so-called photoperiodic counter, which accumulates information from the photoperiodic clock (Vaz Nunes and Veerman, 1982, 1986; Veerman and Vaz Nunes, 1987; Veerman, 2001; Veerman and Veenendaal, 2003).

Although abundant phenomenological data on photoperiodic time measurement have been accumulated, information on the relationship between the development and diapause is still scant. Recently, Suzuki et al. (2007) reported a developmental delay in diapausing females of *T. urticae* under non-24-h light-dark (LD) cycles. Here, the relationship between development and diapause under 24-h LD cycles was examined in a population of *T. urticae* bred in Japan.

Materials and methods

Rearing of T. urticae

The founder population of *T. urticae* was collected from an apple (*Malus pumila* Mill. cv. Fuji) orchard in Akita, Japan (39°15′N) in 2001. In the laboratory, the offspring populations were reared on kidney bean leaves (*Phaseolus vulgaris* L.) under LD 16:8 using white fluorescent lamps at 25°C. A fresh bean leaf onto which 30 adult females were placed was kept on water-soaked cotton in a plastic petri dish (diameter, 9 cm; depth, 2 cm). The adults were allowed to lay eggs for 12 h under continuous darkness (DD) at 25°C. The eggs were maintained for 5 days under the same environmental conditions.

Experimental device

The experimental device was designed according to the techniques described by Suzuki et al. (2007) with some modifications. *T. urticae* eggs were reared in aluminum boxes (20 × 20 × 10 cm), which were placed in an incubator (MIR-153; Sanyo Electric Co. Ltd., Osaka, Japan). Each box was equipped with a white light-emitting diode (LED) (NNN28300; Panasonic Electric Works Co. Ltd., Osaka, Japan), wired to an externally mounted data logger/controller (GK100; ESD Co. Ltd., Tokyo, Japan), which regulated the photoperiod. Ventilation was provided by an electric fan (F2510AP; Shicoh Engineering Co. Ltd., Kanagawa, Japan).

Experimental conditions

Larvae that hatched 5 days after egg laying were transferred onto leaf disks (diameter, 10 mm; 1 mite/disk) that were placed on water-soaked cotton in a plastic petri dish. To determine the effect of the photoperiod on development and the induction of diapause, the mites were reared under various photoperiods: LD 0:24 (DD), 1:23, 2:22, 3:21, 4:20, 5:19, 8:16, 11:13, 12:12, 12.5:11.5, 13:11, 14:10, 15:9, 16:8, and 24:0 (continuous light). To determine the stage sensitive to the photoperiod, long-night (LD 8:16) cycles were applied against a background of DD in the larval and protonymphal (LP), larval and deutonymphal (LD), protonymphal and deutonymphal (PD), and total immature (LPD) stages, respectively (5 photoperiods/stage). In all treatments, the light intensity, air temperature, and relative humidity (RH) were set at 2.5 W m^{-2}, 18.0°C, and 70%, respectively. Air temperature was measured with a copper-constantan thermocouple (wire diameter, 0.1 mm). RH was measured with a variable-resistance-type humidity sensor (CHS-UGS; TDK Co., Tokyo, Japan). Measurements were recorded using the data logger/controller.

Observation

To determine the developmental times of the larval, protonymphal, and deutonymphal stages, individual females were observed every 24 h until they reached the adult stage by using a stereomicroscope (SZ-PT; Olympus Co., Tokyo, Japan).

Diapause induction was examined 7 days after adult emergence. Adult females were considered to be in diapause if their body color turned from yellowish-green to uniform deep orange-red on account of the accumulation of carotenoids (Veerman, 1974). The leaf disks were renewed every 5 days.

Data analysis

The differences in the developmental times of the larval, protonymphal, deutonymphal, and total immature stages under each photoperiod were statistically analyzed by one-way analysis of variance (ANOVA) followed by Scheffe *post hoc* test. The statistical significance of the differences in diapause incidence was tested by Fisher's exact test with a Bonferroni correction. A correlation analysis was conducted to determine the relation between the developmental time for each immature stage and the diapause incidence. The statistical significance of the differences in the developmental times of each immature stage between diapausing and non-diapausing females was tested by the Mann-Whitney *U* test. Calculations were performed using SigmaPlot 2001 (SPSS Inc., Chicago, IL) and SPSS 11.5J software (SPSS Japan Inc., Tokyo, Japan).

Results

Difference in developmental times among different photoperiods

Significant differences were detected in the developmental times of all the immature stages under different photoperiods; the order of significance was as follows: deutonymph [F (13, 603) = 62.801, $P < 0.001$] > protonymph [F (13, 603) = 16.983, $P < 0.001$] > larva [F (13, 603) = 9.826, $P < 0.001$] (Table 4.1). Significance was also determined for the total post-embryonic developmental period [F (13, 603) = 56.425, $P < 0.001$].

Table 4.1 Developmental times (mean ± SE) of females of *Tetranychus urticae* in each stage under different light periods.

Light period [h day^{-1}]	*N*	Larva [day]	Protonymph [day]	Deutonymph [day]	Total [day]
1	37	3.5 ± 0.1 abc	3.2 ± 0.1 cde	5.0 ± 0.1 abc	11.7 ± 0.1 def
2	90	3.5 ± 0.1 abc	3.8 ± 0.1 ab	5.0 ± 0.1 abc	11.7 ± 0.1 abcd
3	36	3.6 ± 0.1 abc	4.0 ± 0.0 a	5.3 ± 0.1 ab	12.8 ± 0.1 ab
4	45	3.6 ± 0.1 abc	3.5 ± 0.1 abcd	5.3 ± 0.1 ab	12.5 ± 0.1 abcd
5	37	3.8 ± 0.1 ab	3.9 ± 0.1 ab	5.3 ± 0.1 ab	12.9 ± 0.2 a
8	44	3.8 ± 0.1 ab	3.4 ± 0.1 bcd	5.5 ± 0.1 a	12.7 ± 0.1 ab
11	47	3.1 ± 0.1 c	3.3 ± 0.1 cde	5.4 ± 0.1 a	11.8 ± 0.1 cdef
12	45	3.7 ± 0.1 abc	3.4 ± 0.1 bcd	5.5 ± 0.1 a	12.6 ± 0.1 abc
12.5	37	3.1 ± 0.1 c	3.3 ± 0.1 cde	4.8 ± 0.1 bc	11.2 ± 0.2 f
13	31	4.0 ± 0.1 a	3.6 ± 0.1 abc	4.5 ± 0.1 c	12.1 ± 0.1 bcde
14	38	3.8 ± 0.1 ab	3.5 ± 0.1 abcd	3.9 ± 0.1 d	11.3 ± 0.1 f
15	40	3.9 ± 0.1 a	3.6 ± 0.1 abcd	4.0 ± 0.1 d	11.5 ± 0.1 ef
16	44	3.4 ± 0.1 abc	3.1 ± 0.1 de	3.8 ± 0.1 d	10.4 ± 0.1 g
24	33	3.3 ± 0.1 bc	2.8 ± 0.1 e	3.7 ± 0.1 d	9.8 ± 0.1 g

ANOVA

Developmental period	Factor	*df*	*SS*	*MS*	*F*
Larval	Total	603	209.795		
	Light period	13	37.337	2.872	9.826***
	Error	590	172.458	0.292	
Protonymphal	Total	603	190.800		
	Light period	13	51.955	3.997	16.983***
	Error	590	138.845	0.235	
Deutonymphal	Total	603	403.422		
	Light period	13	234.184	18.014	62.801***
	Error	590	169.238	0.287	
Total	Total	603	789.219		
	Light period	13	437.400	33.646	56.425***
	Error	590	351.818	0.596	

Values in the same column followed by different letters are significantly different at $P < 0.05$ as determined by the Scheffe *post hoc* test after one-way ANOVA (***: $P < 0.001$).

Photoperiodic-response curve for diapause induction

Diapause induction was not observed in any of the females under DD (Fig. 4.1). Although 8% females entered diapause under LD 1:23, the diapause incidence was not significantly different from that observed under DD ($P > 0.05/105$, Fisher's exact test with Bonferroni correction). Under LD 2:22, the incidence of diapause induction was 50%, which was significantly higher than that observed under LD 1:23 ($P < 0.05/105$). With 3–12 h day^{-1} light periods, the incidence of diapause induction exceeded 80% and was significantly higher than that observed under LD 2:22 ($P < 0.05/105$). Under LD 12.5:11.5, the incidence decreased to 54% and was significantly lower than that observed under the 3–12 h day^{-1} light periods (except for the 4 h day^{-1} light period) ($P < 0.05/105$). Under longer light periods, the incidence decreased further (13% under LD 13:11), and diapause induction failed completely when the light period exceeded 14 h day^{-1}.

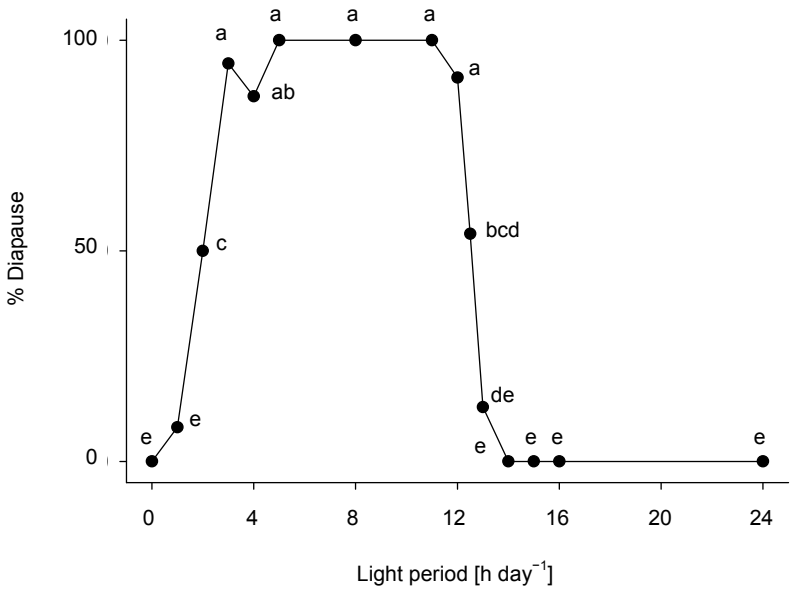

Fig. 4.1 Photoperiodic-response curve for the induction of diapause in *Tetranychus urticae*. Different letters indicate a significant difference at $P < 0.05/105$, as determined by Fisher's exact test with Bonferroni correction. N = 31–90.

Interestingly, under LD 2:22 and 4:20, each female laid 2 and 3 eggs, respectively, though their body color had changed to a uniform deep orange-red. I regarded these females as non-diapausing females.

Relationships between developmental time and diapause incidence

A significant positive correlation was detected between developmental times and diapause incidence in the deutonymphal (R^2 = 0.88, P < 0.0001) (Fig. 4.2a) and total immature stages (R^2 = 0.66, P = 0.011) (Fig. 4.2b). However, no significant correlation was observed between the developmental times and diapause incidence in the larval and protonymphal stages (P > 0.05). Data shown in Table 4.1 and Figure 4.1 were used for these analyses.

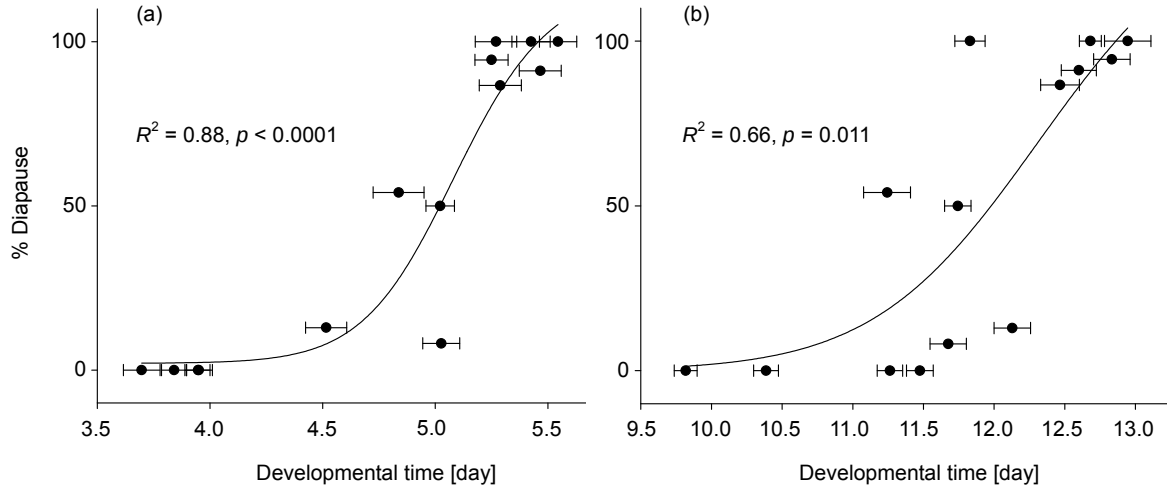

Fig. 4.2 Relationship of the developmental times of the (a) deutonymphal and (b) total immature stages with diapause incidence in *Tetranychus urticae*. Horizontal bars indicate SE of the mean. N = 31–90. Data from Table 4.1 and Figure 4.1 were used.

Difference in developmental times between diapausing and non-diapausing females

The developmental times under LD 2:22 and 12.5:11.5, wherein almost half the females entered diapause, were compared between diapausing and non-diapausing females. Under LD 2:22, the developmental times of the protonymphal, deutonymphal, and total immature stages were significantly longer in the diapausing females than in the non-diapausing females ($P < 0.001$, Mann-Whitney U test) (Fig. 4.3a). By contrast, the developmental times of the larval stage were not significantly different ($P > 0.05$). Under LD 12.5:11.5, significant differences were detected in the developmental times of the deutonymphal ($P < 0.001$) and total immature stages ($P < 0.01$), but not in those of the larval and protonymphal stages ($P > 0.05$) (Fig. 4.3b).

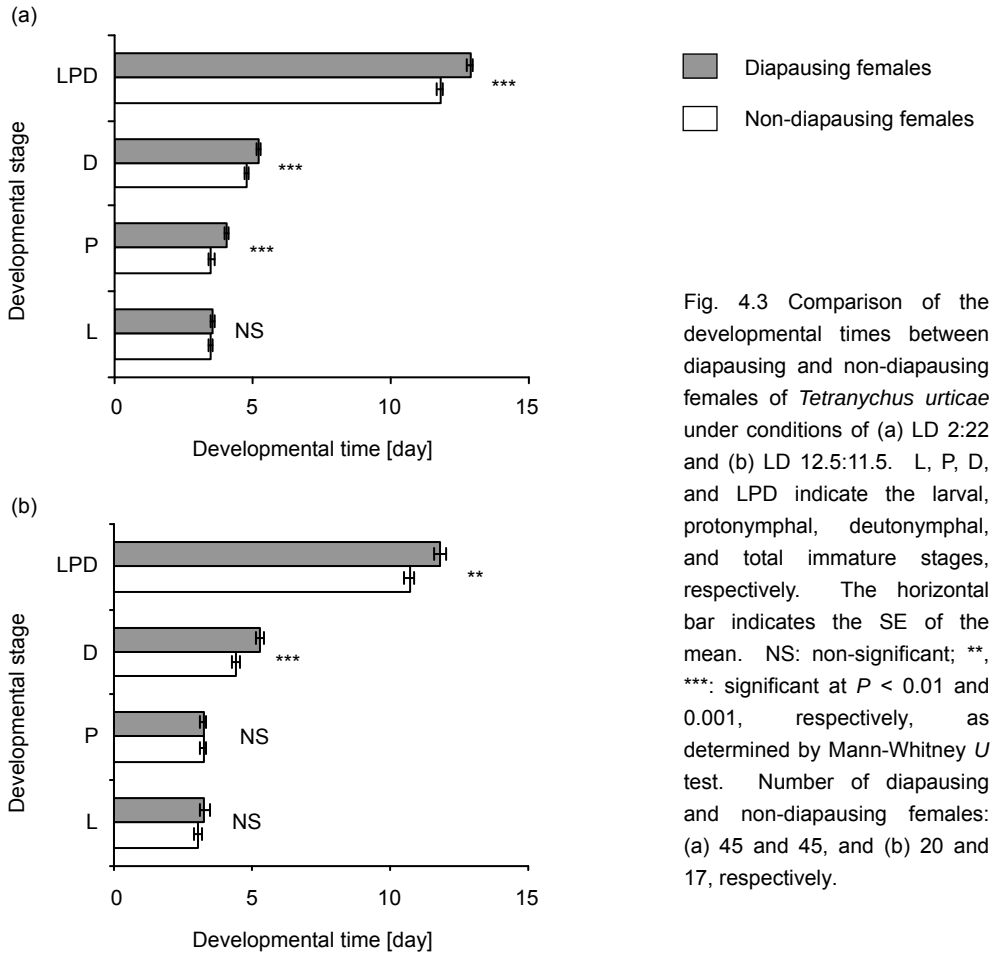

Fig. 4.3 Comparison of the developmental times between diapausing and non-diapausing females of *Tetranychus urticae* under conditions of (a) LD 2:22 and (b) LD 12.5:11.5. L, P, D, and LPD indicate the larval, protonymphal, deutonymphal, and total immature stages, respectively. The horizontal bar indicates the SE of the mean. NS: non-significant; **, ***: significant at $P < 0.01$ and 0.001, respectively, as determined by Mann-Whitney U test. Number of diapausing and non-diapausing females: (a) 45 and 45, and (b) 20 and 17, respectively.

Stages sensitive to photoperiod

The diapause incidence under DD was 0% (Fig. 4.4), which is identical to the result shown in Figure 4.3. When long-night (LD 8:16) cycles were applied against a background of DD to the LP, LD, and PD stages, 35%, 59%, and 67% of females, respectively, entered diapause. These values were significantly higher than those obtained under DD ($P < 0.05/10$, Fisher's exact test with Bonferroni correction). No significant differences in diapause incidence ($P > 0.05/10$) were detected among the LP, LD, and PD treatments. The diapause incidence increased to 80% when long-night cycles were applied during the LPD stages. This incidence was significantly higher than that obtained under DD and under the LP treatment ($P < 0.05/10$).

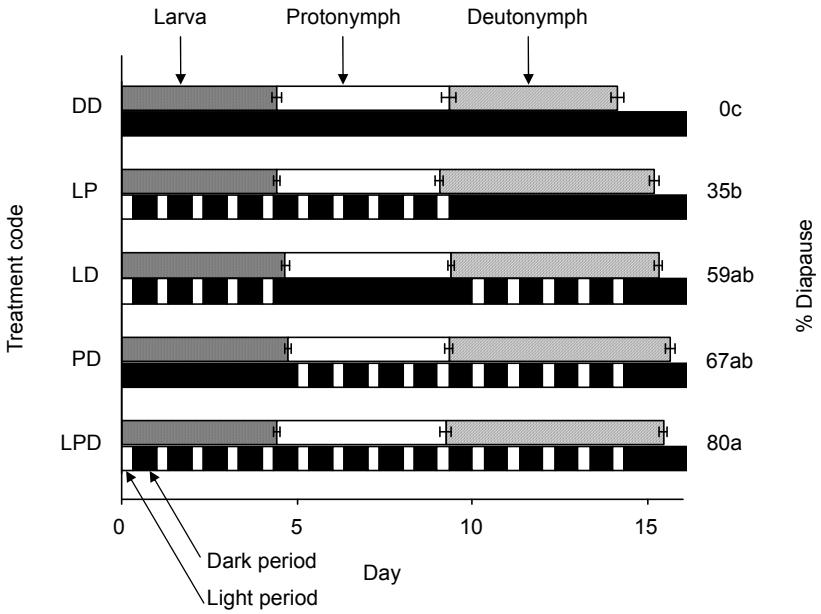

Fig. 4.4 Effect of the duration of photoperiod on diapause induction in *Tetranychus urticae*. DD indicates continuous darkness. Long-night (LD 8:16) cycles were applied against a background of DD in the larval and protonymphal (LP), larval and deutonymphal (LD), protonymphal and deutonymphal (PD), and total immature (LPD) stages, respectively (5 photoperiods/stage). The horizontal bar indicates the SE of the mean. Different letters followed by the percentage of diapause induction indicate a significant difference at $P < 0.05/10$, as determined by Fisher's exact test with Bonferroni correction. Number of females under DD, LP, LD, PD, and LPD: 24, 31, 39, 30, and 30, respectively.

Discussion

Photoperiodic-response curve for diapause induction

The photoperiodic-response curve had two critical daylengths (CDLs) that were detected in the long and short light periods (Fig. 4.1); I have designated these as CDL_{long} and CDL_{short}, respectively. The CDL_{long} (12.5 h) for the Akita (39°N) population used in the present study was longer than that for the *T. urticae* populations from Tashkent (42°N) (11 h), Tiflis (42°N) (11 h), Krasnodar (45°N) (12 h), and Tottori (35°N) (12 h), and shorter than that for the populations from Sapporo (43°N) (13 h), Littlehampton (51°N) (13–14 h), Cambridge (52°N) (13–14 h), Voorne (52°N) (14 h), and Leningrad (60°N) (17 h) (Parr and Hussey, 1966; Veerman, 1977; Uchida, 1980; Gotoh, 1986). Thus, the CDL_{long} tends to be longer as the latitude increases. CDL_{long} is called the ecological threshold (Danilevsky et al., 1970). CDL_{short} is considered the physiological threshold as it is irrelevant to ecological adaptation, and its significance has not been studied intensively; few reports are available on CDL_{short}. Veerman (1977) demonstrated that the CDL_{short} was 0–1 h in the Voorne population. In the present study, the CDL_{short} was 2 h. Diapause was not detected in females under DD (Figs. 4.1, 4.4); hence, I can infer that a decrease in diapause incidence under the short light period may be attributable to the absence of sufficient photoreception.

Under LD 2:22 and 4:20, females that had atypical characteristics were observed: they were deep orange-red (typical of diapausing females), but they laid a few eggs. These findings were probably caused by contradicting signals to induce diapause and reproduction. This suggests that the females of *T. urticae* can switch from oviposition to diapause in the early adult stage depending on the environmental conditions.

Relationship of development with diapause

Photoperiodic regulation of development is known to occur in some species, especially crickets. Delay in nymphal growth has been observed under long-day conditions and found to be accompanied by larger adult size in *Velarifictorus* (*Scapsipedus*) *aspersus* (Saeki, 1966), *Teleogryllus yezoemma* (Masaki, 1966), *Teleogryllus emma* (Masaki, 1967), and *Pteronemobius taprobanensis* (Masaki, 1978). Unlike the above-mentioned crickets, the post-embryonic development of *T. urticae* was inhibited under short-day conditions, except under LD 1:23 and 2:22 (Table 4.1). The delay observed in the present study was probably associated with diapause-inducing signals because of the following reasons. (1) Most females entered diapause under these photoperiodic conditions (Fig. 4.1). (2) A significant correlation between the developmental time and diapause incidence was detected in the deutonymphal and total immature stages (Fig. 4.2). (3) The delay in development, particularly in the deutonymphal stage, was observed in diapausing females (Fig. 4.3). These relations between development and diapause under 24-h LD cycles were comparable with those observed under non-24-h LD cycles (Suzuki et al., 2007). This suggests that developmental delay is not directly regulated by the LD cycle, but by physiological changes that induce diapause. The prolongation of the developmental period in the immature stages under diapause-inducing conditions might contribute to an increase in growth for overwintering. Further studies on the photoperiodic regulation of the female body size are required to test this hypothesis. However, the developmental delay might merely have been due to metabolic decline, which occurred in females that subsequently entered diapause.

Photoperiodic sensitivity during development

Parr and Hussey (1966) and Veerman (1977) determined the developmental stages of *T. urticae* that are most sensitive to photoperiods. They reported that the most sensitive stage was the protonymphal (Veerman, 1977) or both protonymphal and deutonymphal (Parr and Hussey, 1966). Moreover, Parr and Hussey (1966) and Veerman (1977) showed that the larval stage is also sensitive to photoperiods. My results slightly differ from those of Veerman (1977). I regarded the deutonymph as the most sensitive stage because diapause incidence was high when long-night cycles were applied against a background of DD in the deutonymphal stage (Fig. 4.4). The larval and protonymphal stages were also sensitive to photoperiods; however, the order of the sensitivity was as follows: larva < protonymph < deutonymph. This result agrees with that of Parr and Hussey (1966) and indicates that not only the physiological changes accompanying developmental delay but also photoperiodic time measurement can be mainly conducted in the deutonymphal stage.

Concluding remarks

Many theoretical models are available for interpreting the photoperiodism data in *T. urticae*. In the near future, photoperiodism in *T. urticae* should be studied using physiological, biochemical, and molecular investigations to improve our understanding of the photoreception mechanism, the molecular mechanism of the circadian system and its involvement in diapause induction, and the hormonal mechanism of diapause regulation. However, further phenomenological data on photoperiodism in *T. urticae* are required.

The present study revealed that the deutonymphal development is inhibited by diapause-inducing signals and that the deutonymph exhibits high photoperiodic sensitivity, suggesting that the deutonymphal stage is critical for the photoperiodic regulation of reproduction or diapause in *T. urticae*.

Chapter 5

Action spectrum for the suppression of arylalkylamine *N*-acetyltransferase activity in the two-spotted spider mite *Tetranychus urticae*

Abstract

An action spectrum was obtained for the suppression of arylalkylamine *N*-acetyltransferase (NAT) activity in the two-spotted spider mite *Tetranychus urticae* by irradiating the mite with monochromatic lights of various wavelengths using the Okazaki Large Spectrograph at the National Institute for Basic Biology, Okazaki, Japan. Fluence-response curves were obtained for wavelengths between 300 and 650 nm by irradiating the mite for 4 h d^{-1}. The samples were frozen after the third exposure. A negative correlation between the logarithmic fluence rate and NAT activity was detected in the range of 0.01–1 μmol m^{-2} s^{-1} for wavelengths between 300 and 500 nm and in the range of 0.1–10 μmol m^{-2} s^{-1} for wavelengths between 550 and 650 nm. The constructed action spectrum indicated that the photoreceptors mediating the circadian and/or photoperiodic systems might be ultraviolet (UV)-A- and blue-type photoreceptors with absorption peaks at 350 and 450 nm.

Introduction

The two-spotted spider mite *Tetranychus urticae* is distributed worldwide and considered a serious pest for a wide variety of crops (van de Vrie et al., 1972), and it acts as a typical *r*-strategist (Sabelis, 1985b). The *r* is elevated based on a short developmental period and high fecundity enforced by life-history characteristics to produce successive generations. Growth rate and photoperiodism are important determinants in the life history of this mite.

Veerman and his colleagues conducted phenomenological investigations on the mechanism underlying the photoperiodic determination of diapause in *T. urticae*. They proposed an empirical model based on 3 basic assumptions: (1) a non-repetitive long-night measuring photoperiodic timer, which may either be a real hourglass or a rapidly damped oscillator; (2) since the Nanda-Hamner protocol (Nanda and Hamner, 1958) produced rhythmic fluctuations in diapause induction, the circadian system is somehow involved in the photoperiodic response; and (3) the existence of the so-called photoperiodic counter, which integrates photoperiodic "information" (Vaz Nunes and Veerman, 1982, 1986; Veerman and Vaz Nunes, 1987; Veerman, 2001; Veerman and Veenendaal, 2003). However, several aspects of basic knowledge, particularly the hormonal mechanism of diapause regulation, the molecular mechanism of the circadian system and its involvement in photoperiodism, and the photoreception mechanism remain unclarified.

Arylalkylamine *N*-acetyltransferase (NAT; EC 2.3.1.87) catalyzes the *N*-acetylation of arylalkylamines with acetyl-CoA. In vertebrates, NAT is the penultimate and rate-limiting enzyme in the production of the pineal hormone melatonin (*N*-acetyl-5-methoxytryptamine). Melatonin is an indoleamine that is synthesized

from serotonin (5-hydroxytryptamine, 5-HT) via *N*-acetylation catalyzed by NAT and *O*-methylation catalyzed by 5-hydroxyindole-*O*-methyltransferase (HIOMT; EC 2.1.1.4) (Klein and Weller, 1970).

In many vertebrate systems, the output signals of the biological clocks are transduced to the synthesis and release of melatonin. NAT activity exhibits circadian rhythm in the vertebrate pineal gland, which regulates the level of melatonin (Ganguly et al., 2002). Moreover, melatonin is an endocrine token of night, and the amount of melatonin synthesized serves as a timer that controls the seasonal physiology in some cases (Reiter, 1980; Bartness et al., 1993; Lincoln and Clarke, 1994).

Insect NAT (iNAT) plays important roles in the sclerotization of the cuticle and inactivation of monoamine neurotransmitters (Smith, 1990). iNAT (also referred to as DAT) converts dopamine to *N*-acetyldopamine—a tanning agent for the cuticle (Andersen, 1985). In vertebrates, monoamine neurotransmitters are mainly metabolized by monoamine oxidase (MAO; EC 1.4.3.4). In the nervous tissues of insects, however, MAO activity is suggested to be weak or absent (Sloley, 2004). Instead, iNAT is considered a major enzyme involved in the catabolism of monoamines (Dewhurst et al., 1972; Downer and Martin, 1987; Sasaki and Nagao, 2001).

It is unclear whether the synthesis of melatonin in insects follows a pathway similar to that in vertebrates. NAT and HIOMT activities have been detected in insects. The iNAT proteins have been purified from the fruit fly (*Drosophila melanogaster*) (Hintermann et al., 1995; Amherd et al., 2000), American cockroach (*Periplaneta americana*) (Ichihara et al., 1997, 2001), and silkworm (*Bombyx mori*) (Tsugehara et al., 2007); however, HIOMT has not been purified or cloned in insects. In *D. melanogaster*, iNAT activity is not rhythmic in the head although the melatonin concentration fluctuates to exhibit a daily rhythm (Hintermann et al., 1996).

Alternatively, in *P. americana*, the iNAT activity and melatonin concentration in the brain exhibit day/night fluctuations (Bembenek et al., 2005). In *B. mori*, the iNAT activity and melatonin concentration in the head show day/night fluctuations (Itoh et al., 1995), and HIOMT activity exhibits circadian rhythm (Itoh et al., 1997).

No report is available on the investigation of mite NAT (mNAT) and its role in photoperiodism. *T. urticae* has been intensively used in studies on photoperiodism, but its small size has been a limitation in further molecular or physiological investigations. The NAT activity assay is very sensitive and could serve as a tool for monitoring the photoperiodic or circadian clock. Therefore, I investigated the action spectrum for phototransduction to mNAT. The action spectrum obtained should match the absorption characteristics of the putative photoreceptor (Schäfer and Fukshansky, 1984; Coohill, 1991; Holmes, 1997).

Materials and Methods

Laboratory culture of T. urticae

The founder population of *T. urticae* was collected from an apple (*Malus pumila* Mill. cv. Fuji) orchard at Akita, Japan (39°15′N) in 2001. The offspring populations were maintained in the laboratory on kidney bean leaves (*Phaseolus vulgaris* L.) under long-day (LD 16:8) conditions provided by white fluorescent lamps at 25°C. A total of 150 adult females emerged from the teleiochrysalis stage within 2 d; they were then placed onto a fresh bean leaf kept over water-soaked cotton in a plastic petri dish (diameter, 9 cm; depth, 2 cm).

Monochromatic light irradiation

To determine the action spectrum for the suppression of mNAT activity, *T. urticae* was exposed under the Okazaki Large Spectrograph (OLS) (Watanabe et al., 1982) at the National Institute for Basic Biology, Okazaki, Japan. Experimental design was in accordance with that of Kräbs et al. (2004). Monochromatic light was provided by a large spectrograph equipped with a 30-kW xenon arc lamp (Ushio Electric Co., Tokyo, Japan). The light beam was first reflected by a plane mirror and then a condensing mirror. After reflection by a diffraction grating, the light beam passed into the irradiation room. In petri dishes placed at various wavelengths between 300 and 650 nm, *T. urticae* samples were placed beneath a mirror that reflected the monochromatic light beam directly onto them. Figure 5.1 shows the spectral distributions when the photon flux was set at 100 μmol m^{-2} s^{-1}.

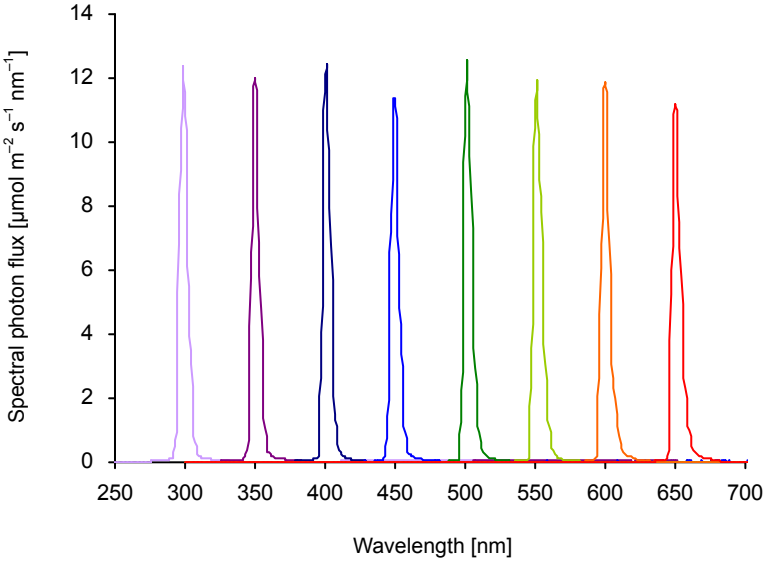

Fig. 5.1 Spectral distributions of the treatments when the photon flux was set at 100 μmol m^{-2} s^{-1}.

Short-cutoff filters (Hoya Co., Tokyo, Japan) were placed above the petri dishes. Fluence rates examined at specific wavelengths (Table 5.1) were adjusted with neutral-density filters (Fujitok Co., Tokyo, Japan) and were measured with a photon-flux density meter (QTM-101; Monotech Inc., Saitama, Japan). Samples were treated in a light-dark cycle (LD 4:20) under monochromatic light with different fluence rates and in continuous darkness (DD) for 3 d; they were then frozen and stored at −80°C.

Table 5.1 Fluence rates at each wavelength used to calculate the action spectrum for the suppression of mNAT activity in *T. urticae*. The tested conditions are indicated by a "+" sign.

Wavelength [nm]	Fluence rate [μmol m^{-2} s^{-1}]			
	0.01	0.1	1	10
300	+	+	+	
350	+	+	+	
400	+	+	+	
450	+	+	+	
500	+	+	+	
550		+	+	+
600		+	+	+
650		+	+	+

NAT activity assay

The NAT activity assay was performed according to Deguchi and Axelrod (1972) and Ichihara et al. (2001) with some modifications. Samples (10 mites/tube) were homogenized by sonication in a homogenization solution (pH 7.4; 20 mM HEPES, 20 mM NaOH, 1 mM ethylenediaminetetraacetic acid (EDTA)-2Na, 1 mM dithiothreitol

(DTT), 100 μg mL^{-1} phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, 0.01% Nonidet P-40, 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin, and 1 μg mL^{-1} pepstatin A). The homogenized samples were centrifuged at 8000 × *g* for 15 min at 4°C. The supernatant was used as a crude enzyme solution. In the standard procedure, the reaction mixture consisted of 2 μL of the crude enzyme solution, 2 μL of 5-HT as the substrate, 2 μL of 0.4 mM acetyl-CoA containing 0.1 mM [^{14}C] acetyl-CoA (specific activity, 2.0 TBq mol^{-1}; Moravek Biochemicals) and 0.3 mM cold acetyl-CoA (hot/cold = 1:3), and 14 μL of Clark and Lubs solution (pH 7.6) (Clark and Lubs, 1917) in a final volume of 20 μL in a polypropylene tube. After incubation at 37°C for 2 min, the reaction was terminated by the addition of 100 μL 5% acetic acid. Liquid scintillation cocktail {(volume, 1 mL) (4 g L^{-1} 2,5-diphenyloxazole (PPO) and 0.1 g L^{-1} 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP)} in toluene/isoamyl alcohol (7:3 v/v) was added to the reaction mixture. The mixture was vortexed for 30 s and centrifuged at 800 × *g* for 3 min. The radioactivity in the organic layer was measured using a liquid scintillation counter (LSC-700; Aloka Co. Ltd., Tokyo, Japan). mNAT activity was estimated by subtracting the activity of the minus-enzyme control from the activity measured.

Protein measurement

The protein content was determined according to Bradford (1976) by using bovine serum albumin as the standard (B4287; Sigma, St. Louis, MO). The values are provided as the mean of duplicate determination.

Calculation of the action spectrum

The reciprocal of the fluence rate required for 80% suppression of mNAT activity under DD was calculated from the fluence response curves and plotted against the wavelength (for overview, see Schäfer and Fukshansky, 1984; Holmes, 1997; Shropshire, 1972).

Data analysis

For mNAT activity, the mean values and standard errors were calculated from 5 replicates. The statistical significance of the differences between mNAT activity under DD and that suppressed by the exposure to different fluence rates at a specific wavelength was tested by one-way analysis of variance (ANOVA) followed by Dunnett's test (Dunnett, 1955). Calculations were performed using the statistical package for the social sciences (SPSS) 11.5J software (SPSS Japan Inc., Tokyo, Japan).

Results

Quantum effect

The mNAT activities on exposure were significantly lower than those obtained under DD even when the photon flux was as low as 0.01 μmol m^{-2} s^{-1} at 350 ($P < 0.001$), 450, and 500 nm ($P < 0.05$) (Fig. 5.2).

At 300 and 400 nm, the differences between the mNAT activities at a photon flux of 0.01 μmol m^{-2} s^{-1} and those obtained under DD were not significant ($P > 0.05$). However, at photon flux of 0.1 and 1 μmol m^{-2} s^{-1}, the mNAT activities decreased at the abovementioned wavelengths and were significantly lower than those obtained under

DD ($P < 0.01$).

At 550 nm, the mNAT activity decreased only at a photon flux of 10 μmol m^{-2} s^{-1}. However, *T. urticae* could not sense this wavelength at a photon flux below 1 μmol m^{-2} s^{-1} at which the difference between mNAT activity at 550 nm and that determined under DD was not significant ($P > 0.05$).

At 600 and 650 nm, the differences between the mNAT activities and those determined under DD were not significant ($P > 0.05$) even if the photon flux was as high as 10 μmol m^{-2} s^{-1}.

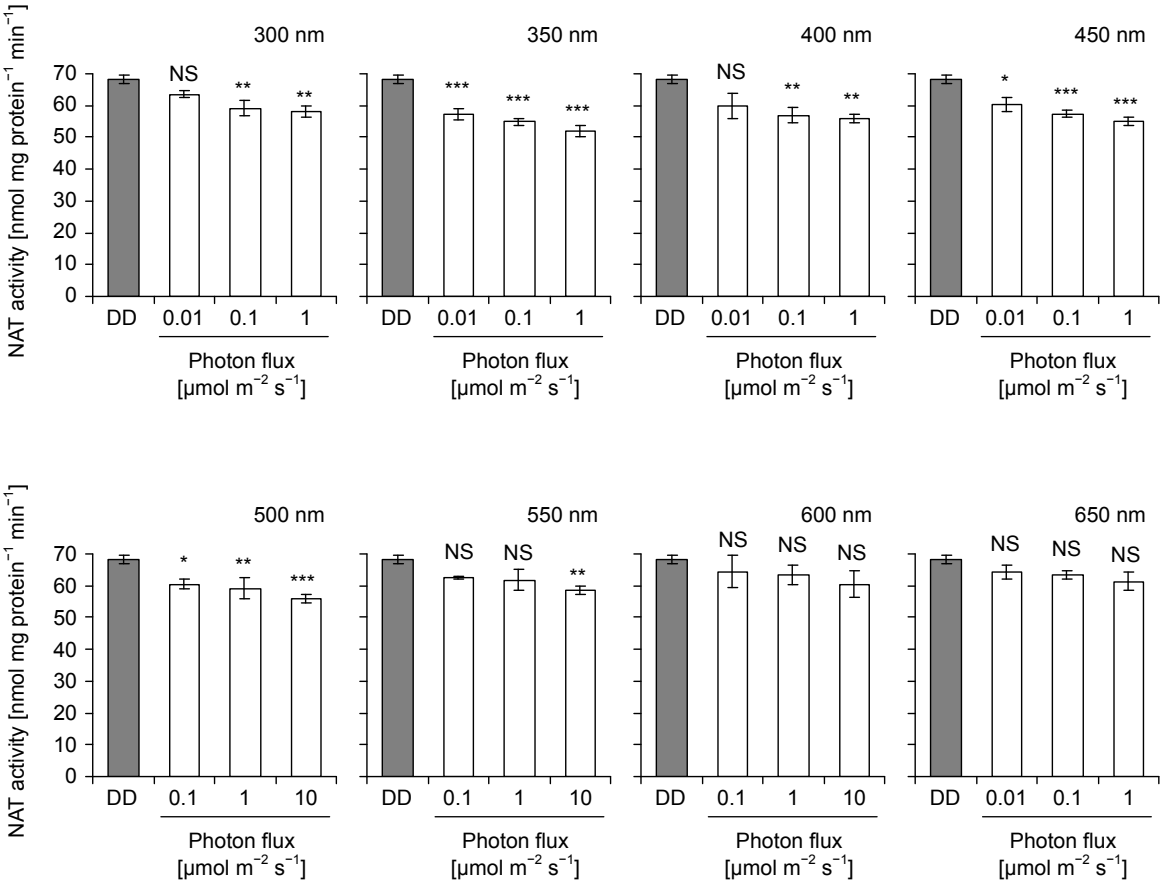

Fig. 5.2 Comparison of the mNAT activity in *Tetranychus urticae* under DD (continuous darkness) with that under each photon flux at all wavelengths. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: not significant by Dunnett's test. Vertical bars indicate ±SE of 5 experiments; $N = 10$.

Dose-response curves

A negative correlation between the logarithmic fluence rate and mNAT activity was detected for wavelengths between 300 and 500 nm in the fluence rate range of 0.01–1 µmol m^{-2} s^{-1} and for wavelengths between 550 and 650 nm in the fluence rate range of 0.1–10 µmol m^{-2} s^{-1} (Fig. 5.3).

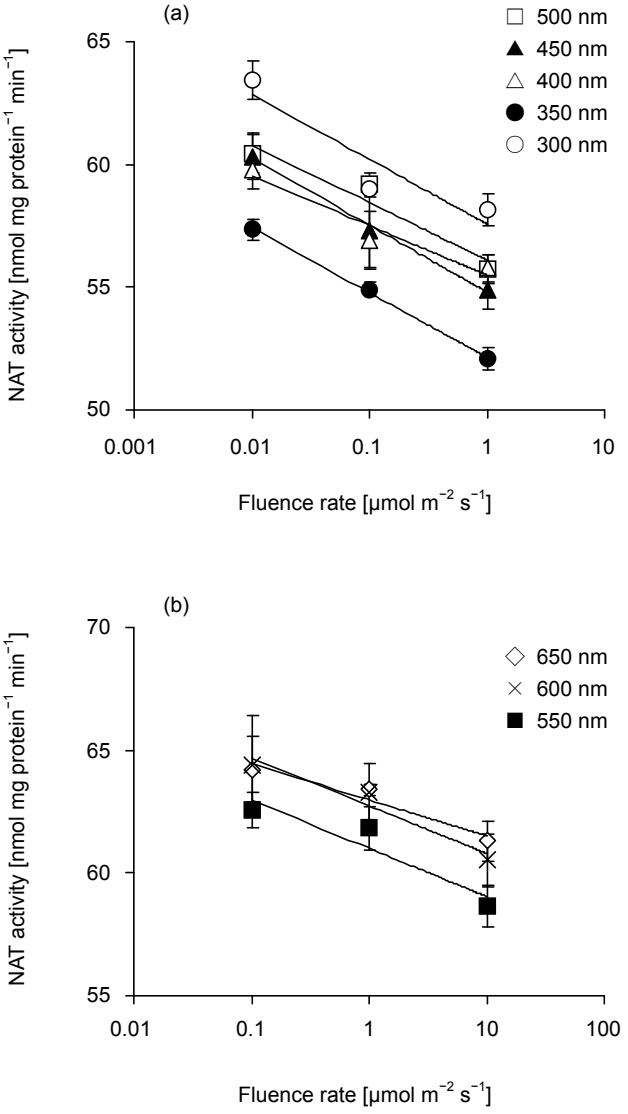

Fig. 5.3 Fluence-response curves for mNAT activity in *Tetranychus urticae* exposed to monochromatic lights with different fluence rates for 4 h d^{-1}. Vertical bars indicate ±SE of 5 experiments; *N* = 10; (a) 300–500 nm; (b) 550–650 nm.

Action spectrum

The action spectrum (Fig. 5.4) for the dose-response curves (Figs. 5.2, 5.3) was determined by plotting the relative reciprocal values of the fluence rate required for 80% suppression of the mNAT activity under DD (68 nmol mg protein^{-1} min^{-1}).

The main maximum of the action spectrum is at 350 nm and the second maximum, at 450 nm.

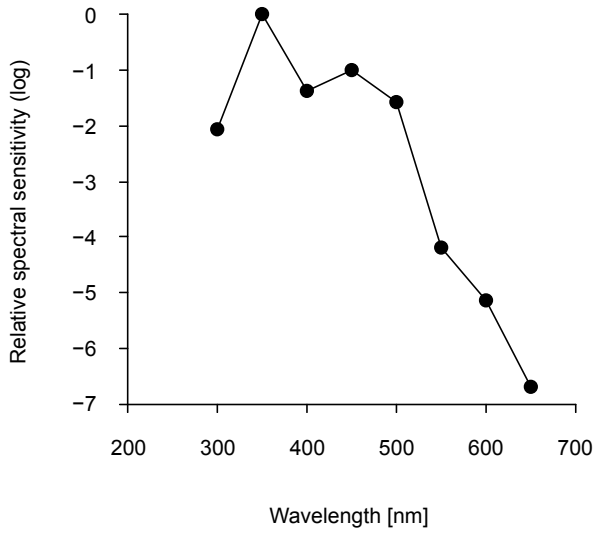

Fig. 5.4 Action spectrum for the suppression of mNAT activity in *Tetranychus urticae*. The relative spectral sensitivity, i.e., the reciprocal of the fluence rate required for 80% suppression of mNAT activity under DD (68 nmol mg protein^{-1} min^{-1}), was calculated (from data in Figs. 5.2 and 5.3) and plotted against the wavelength.

Discussion

In the past, many authors have investigated the wavelength-dependent suppression of NAT activity in vertebrates. Deguchi (1981) revealed that in the pineal gland of chickens, wavelengths of 500 and 520 nm are the most effective in suppressing NAT activity, and the existence of a rhodopsin-like photoreceptor was suggested. Okano et al. (1994) have isolated the photoreceptor cDNA clone and named it "pinopsin."

Insects catabolize monoamine neurotransmitters mainly by *N*-acetylation (Dewhurst et al., 1972; Downer and Martin, 1987; Sasaki and Nagao, 2001) rather than by the activity of MAO, which is the main catabolic enzyme of monoamines in vertebrates. However, with regard to insects, information on the nature of iNAT-regulating mechanisms, iNAT involvement in the circadian and photoperiodic systems, and the putative photoreceptor remains limited.

In *T. urticae*, the exact photoperiodic mechanism remains unclear because of the small size of this mite, although abundant data regarding photoperiodism have been accumulated. In *T. urticae*, the mNAT activity showed day/night fluctuations: the activity was suppressed in the light period with white light (unpublished). These fluctuations are similar to those of the daily rhythm of iNAT activity in *P. americana* (Bembenek et al., 2005) and *B. mori* (Itoh et al., 1995). However, it is unclear which wavelength is effective for suppression of mNAT activity since white light includes various wavelengths. Therefore, the present study attempted to clarify the mechanism of photoreception by the wavelength-dependent suppression of mNAT activity. In this study, the main maximum of the action spectrum obtained is at 350 nm and the second maximum, at 450 nm. This result is not comparable with Deguchi's results with the

chicken pineal gland (Deguchi, 1981). The presence of photoreceptors different from pinopsin (Okano et al., 1994) has been inferred.

Veerman (2001) has suggested that the photoperiodic clock in insects and mites most probably operates as a non-circadian hourglass with an opsin-based photoreceptor that is coupled with a carotenoid-derived chromophore, and that the circadian system plays certain roles in insect and mite photoperiodism, similar to that in the case of the Nanda-Hamner rhythm (controlled by a different photoreceptor). The photoreceptor for circadian entrainment might be a flavin based cryptochrome (CRY), as demonstrated in *Drosophila* (Hall, 2000; Van Gelder, 2002).

In *T. urticae*, the Nanda-Hamner rhythm (which has a period of free-running rhythm, the so-called τ value) is only 20 h, and the desynchronization of τ with an LD cycle (T) disturbed the photoperiodic induction of diapause (Veerman and Vaz Nunes, 1980). Veerman and Veenendaal (2003) reported that *T. urticae* exhibited strong sensitivity in the photoperiodic response to orange-red light (>580 nm) in T = 20 h but not in T = 24 h. This might be due to the desynchronization of τ = 20 h with T = 24 h in orange-red light, i.e., the absence of photoreception by the photoreceptor for circadian entrainment. Furthermore, Suzuki et al. (Suzuki, 2008a) revealed that in *T. urticae*, the order of sensitivity in the photoperiodic response in T = 24 h under visible light wavelengths was as follows: blue (475 nm) > green (572 nm) > orange (612 nm), and no sensitivity to red light (658 nm). The action spectrum obtained in this study is comparable with the sensitivity of the photoperiodic response. Moreover, the action spectrum is similar to the absorption spectra of *Drosophila* CRY (dCRY) (Berndt et al., 2007). These results indicate that the mNAT of *T. urticae* may be involved in photoperiodism, particularly in circadian elements, and that its activity may be suppressed by the photoreception of the photoreceptor, which is possibly CRY.

Although the dCRY hardly absorbs light above 500 nm (Berndt et al., 2007), mNAT activity was suppressed at 500 and 550 nm in the present study. Moreover, negative correlations between the logarithmic fluence rate and mNAT activity were detected in the dose-response curves at 600 and 650 nm. If the fluence rate increases further (>10 μmol m^{-2} s^{-1}), suppression of mNAT might also be observed even at such wavelengths. To explain the difference at long wavelengths, identification and characterization of CRY and also the possibility of the other short-wave photoreceptors in *T. urticae* being involved should be investigated.

In rats, circadian clock-related proteins CLOCK/BMAL1 heterodimers bind to E-box elements in the promoter of *NAT* gene and activate its expression (Chen and Baler, 2000). A similar mechanism of *NAT* expression is found in chickens (Chong et al., 2000). In *B. mori*, distributions of immunoreactivities of CYCLE (a homolog of BMAL1 in insects) almost match that of iNAT (Sehadová et al., 2004). This suggests a possibility that transcription of iNAT is regulated by CYCLE (and/or CLOCK) in insects. In mites, no report is available on the molecular investigation of the circadian system. However, mNAT may be a key to investigating circadian and/or photoperiodic mechanisms. The present study shows that mNAT can be utilized as the first internal marker to monitor circadian and/or photoperiodic mechanism in mites.

Chapter 6

UV sensitivity in relation to diapause in the two-spotted spider mite, *Tetranychus urticae*

Abstract

Sensitivity to ultraviolet (UV) radiation in non-diapausing and diapausing adult females of the two-spotted spider mite *Tetranychus urticae* was examined by UV-C (250 nm), UV-B (300 nm), and UV-A (350 nm) exposure; the Okazaki Large Spectrograph at the National Institute for Basic Biology, Okazaki, Japan, was used. In non-diapausing females, the median effective dose (ED_{50}) values for 50% mortality plus escape rate were 21 (UV-C) and 104 kJ m^{-2} (UV-B), and those for 50% oviposition rate in the control-treated mites (continuous darkness) were 6.2 (UV-C) and 41 kJ m^{-2} (UV-B). However, UV-A had no significant effects on the mortality and oviposition rates. The ED_{50} values for UV-B are comparable to the natural UV radiation observed for 2–5 days in summer when these mites inhabit the underside of leaves with dense vegetation. Therefore, they may use leaves as an UV-B-cut filter to avoid the deleterious effects of UV-B. In diapausing females, low mortality rates were observed even at high doses of all the UV radiations, and more than half of the diapausing females escaped even at low doses of all the UV radiations. The orange body color observed in diapausing females is known to result from accumulation of carotenoids, a scavenger for UV-induced reactive oxygen species; this might explain the low mortality rate observed in diapausing females. Thus, diapausing females may have at least 2 means of survival: (1) emigration to UV-free environments and (2) carotenoid accumulation to overcome the deleterious effects of UV radiation during winter in the absence of leaves.

Introduction

Ultraviolet (UV) radiation directly damages DNA and is absorbed by some coenzymes and pigments in vivo; UV absorption raises them to an excited state, and the excitation energy is finally transferred to H_2O molecules, yielding reactive oxygen species (ROS). It has been suggested that UV-induced ROS damage important intra- and extracellular components such as lipids, lipid membranes, nucleic acids, and proteins (Jurkiewicz and Buettner, 1994; Shindo et al., 1994). Such UV/ROS-induced damage would be lethal to mites because of their small size; i.e., their body surface area per weight is large. Therefore, artificial UV irradiation could prove to be a promising non-chemical measure for reducing the population of the two-spotted spider mite (*Tetranychus urticae*), which is distributed worldwide and causes serious damage to a wide variety of crops (van de Vrie et al., 1972).

There is abundant information on the response to light, particularly photoperiodism, in *T. urticae*. Only adult females enter diapause in order to survive during winter by sensing long-night conditions, and diapausing females, which exhibit a bright orange body color in this period, do not show feeding and oviposition (Veerman, 1985).

Response to UV radiation in *T. urticae* has been investigated with regard to behavior (McEnroe and Dronka, 1966; Naegele et al., 1966; Barcelo and Calkins, 1980; Barcelo, 1981), resistance (Barcelo, 1981), and light-dependent enzyme activity (Suzuki et al., 2008b, 2009). However, these experiments were conducted with non-diapausing females, and data on the response of diapausing females to UV radiation still remains limited.

The present study investigates the difference in sensitivity to UV radiation between non-diapausing and diapausing adult females and discusses the phenological adaptation to UV radiation in *T. urticae*.

Materials and Methods

Laboratory culture of T. urticae

The founder population of *T. urticae* was collected from an apple (*Malus pumila* Mill. cv. Fuji) orchard located in Akita, Japan (39°15′N), in 2001. The offspring populations were maintained in the laboratory on kidney bean leaves (*Phaseolus vulgaris* L.) under long-day (LD 16:8) conditions provided by white fluorescent lamps at 25°C. The adult females and males that emerged from the teleiochrysalis stage within 3 days were used in the following UV resistance experiment.

In order to obtain diapausing females, adult females were placed on a fresh bean leaf that was positioned on water-soaked cotton in a plastic petri dish (diameter, 9 cm; depth, 2 cm); they were kept here for 12 h under continuous darkness (DD) at 25°C for oviposition and removed after eggs were laid. The eggs were maintained for 5 days under the same environmental conditions; then, the larvae that hatched were exposed to short-day (LD 8:16) conditions provided by white light-emitting diodes at 18°C. Diapause induction was determined to occur 7–10 days after adult emergence, and the adult females with uniform orange coloration were used in the following UV resistance experiment.

The non-diapausing and diapausing females were then placed on fresh leaf disks (diameter, 1 cm; 1 individual per disk) kept over water-soaked cotton in the plastic petri dish.

UV irradiation

The effects of UV radiation on the mortality, escape, and oviposition rates in *T. urticae* were determined by using the Okazaki Large Spectrograph (OLS) (Watanabe et al., 1982) at the National Institute for Basic Biology, Okazaki, Japan. Monochromatic light was provided by a large spectrograph equipped with a 30-kW xenon arc lamp (Ushio Electric Co., Tokyo, Japan). The light beam is first reflected by a plane mirror and then by a condensing mirror. After reflection by a diffraction grating, the light beam passes into the irradiation boxes (Fig. 6.1).

In each petri dish placed in the boxes with various wavelengths of 250, 300, and 350 nm, *T. urticae* samples were placed beneath a mirror that reflected the monochromatic light beam directly onto them. Table 6.1 lists the types of treatment, wavelength peak, irradiance, and dosages. Figure 6.2 shows the spectral distributions of the treatments when the irradiance from 200 to 800 nm was set at 100 W m^{-2}.

The irradiance values (0.06–0.6 W m^{-2} for UV-C and 0.2–2.4 W m^{-2} for UV-B and UV-A) at specific wavelengths were adjusted with UV filters (Fujitok Co., Tokyo, Japan) and measured with a radiation sensor (QTM-101; Monotech Inc., Saitama, Japan). Samples were treated under a light–dark cycle (LD 4:20) with monochromatic light of different irradiances for 3 days.

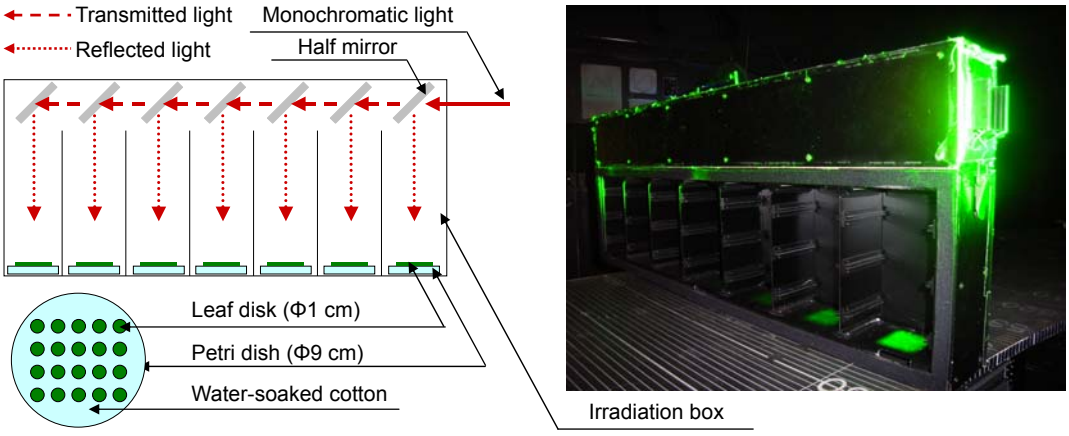

Fig. 6.1 Schematic diagrams and a photograph of the irradiation box. The lower diagram shows a top view of the petri dish.

Table 6.1 Type of UV radiation, wavelength peak, irradiance, and daily-integrated dose.

Type of UV radiation	Wavelength peak [nm]	Irradiance [W m^{-2}]	Daily-integrated dose [kJ m^{-2} day^{-1}]
UV-C	250	0.06	0.9
		0.2	2.9
		0.6	8.6
UV-B	300	0.2	2.9
		0.6	8.6
		2.4	34.6
UV-A	350	0.2	2.9
		0.6	8.6
		2.4	34.6

Exposure time was 4 h per day.

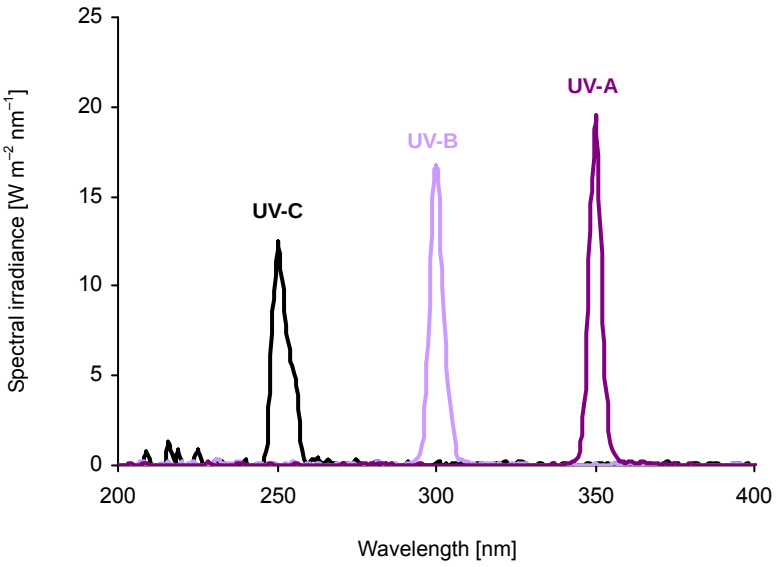

Fig. 6.2 Spectral distributions of the treatments when the irradiance was set at 100 W m^{-2}.

Observations

After irradiation everyday, in the number of non-diapausing and diapausing females who were alive, dead or had escaped and the number of eggs laid in non-diapausing females were observed with a stereomicroscope (SZ-PT; Olympus Co., Tokyo, Japan). Viability or mortality was judged with the help of a brush; individuals that showed no reaction on contact with the brush were regarded as dead. Individuals that had fallen off the leaf disk onto the water-soaked cotton were considered to have escaped. The observation was conducted under red light-emitting diodes that *T. urticae* cannot sense (Suzuki et al, 2008a); thus, the undesirable effects of light were prevented.

Statistical analysis

The statistical significance of the differences between the mortality and escape rates under DD (control) and those on exposure to different irradiances at a specific wavelength was determined by Fisher's exact test. The statistical significance of the differences between the oviposition rate under DD and that on exposure to different irradiances at a specific wavelength was determined by one-way ANOVA and then Dunnett's test. A correlation analysis was conducted to determine the UV-dose response in terms of the mortality, escape, and oviposition rates. The calculations were performed by using SigmaPlot 2001 (SPSS Inc., Chicago, IL) and the SPSS 11.5J software (SPSS Japan Inc., Tokyo, Japan).

Results

Mortality

In non-diapausing females, the differences between the mortality rates in all the UV treatments and those in the control conditions (DD) were not significant on days 1 and 2 ($P > 0.05$, Fisher's exact test); the mortality rates were nearly 0% (Table 6.2). Even on day 3, the mortality rates in the UV-C, UV-B, and UV-A treatments with irradiances below 0.2, 0.6, and 2.4 W m^{-2}, respectively, were not significantly different from those under DD ($P > 0.05$). However, the mortality rates on day 3 were significantly high with UV-C and UV-B irradiances as high as 0.6 W m^{-2} ($P < 0.01$) and 2.4 W m^{-2} ($P < 0.001$), respectively, compared to those under DD.

Table 6.2 Daily changes in the mortality rates in non-diapausing *Tetranychus urticae* adult females under continuous darkness and UV radiation.

Treatment	Irradiance [W m^{-2}]	N^a	Cumulative mortality rate [%]		
			Day 1	Day 2	Day 3
Control (DD)	—	98	1	2	4
UV-C	0.06	60	0^{NS}	0^{NS}	7^{NS}
	0.2	65	0^{NS}	3^{NS}	6^{NS}
	0.6	65	0^{NS}	3^{NS}	18**
UV-B	0.2	60	0^{NS}	2^{NS}	3^{NS}
	0.6	59	2^{NS}	2^{NS}	2^{NS}
	2.4	60	0^{NS}	3^{NS}	32***
UV-A	0.2	60	0^{NS}	0^{NS}	0^{NS}
	0.6	60	0^{NS}	2^{NS}	5^{NS}
	2.4	60	0^{NS}	0^{NS}	0^{NS}

[a]Number of individuals. **: $P < 0.01$, ***: $P < 0.001$, NS: not significant ($P > 0.05$) between the control and UV treatments within the same column by Fisher's exact test, and DD: continuous darkness.

In diapausing females, the differences between the mortality rates in all the UV treatments and those under DD were not significant ($P > 0.05$) even on day 3 (Table 6.3).

Table 6.3 Daily changes in the mortality rates in diapausing *Tetranychus urticae* adult females under continuous darkness and UV radiation.

Treatment	Irradiance [W m^{-2}]	N^a	Cumulative mortality rate [%]		
			Day 1	Day 2	Day 3
Control (DD)	—	72	0	1	1
UV-C	0.06	52	6^{NS}	10^{NS}	10^{NS}
	0.2	57	2^{NS}	4^{NS}	5^{NS}
	0.6	65	0^{NS}	0^{NS}	6^{NS}
UV-B	0.2	50	0^{NS}	4^{NS}	6^{NS}
	0.6	52	0^{NS}	2^{NS}	6^{NS}
	2.4	60	0^{NS}	3^{NS}	5^{NS}
UV-A	0.2	50	0^{NS}	2^{NS}	2^{NS}
	0.6	50	2^{NS}	8^{NS}	8^{NS}
	2.4	59	0^{NS}	3^{NS}	8^{NS}

[a]Number of individuals. NS: not significant ($P > 0.05$) between the control and UV treatments within the same column by Fisher's exact test, DD: continuous darkness.

Escape

In non-diapausing females, the escape rates were significantly high when the UV-C irradiance was as high as 0.6 W m^{-2} on day 2 ($P < 0.001$) and > 0.2 W m^{-2} on day 3 ($P < 0.05$) compared to those under DD (Table 6.4). When the UV-B irradiance was as high as 2.4 W m^{-2}, the escape rates were significantly higher than those under DD on days 2 and 3 ($P < 0.01$). When the UV-A irradiance was as high as 2.4 W m^{-2}, the escape rate was significantly higher than that under DD even on day 1 ($P < 0.01$). A significant difference was also detected between the escape rate with UV-A irradiance as low as 0.2 W m^{-2} and that under DD on days 2 ($P < 0.01$) and 3 ($P < 0.05$).

Table 6.4 Daily changes in the escape rates in non-diapausing *Tetranychus urticae* adult females under continuous darkness and UV radiation.

Treatment	Irradiance [W m^{-2}]	N^a	Cumulative escape rate [%]		
			Day 1	Day 2	Day 3
Control (DD)	—	98	0	0	3
UV-C	0.06	60	0^{NS}	2^{NS}	3^{NS}
	0.2	65	2^{NS}	2^{NS}	12*
	0.6	65	3^{NS}	15***	49***
UV-B	0.2	60	0^{NS}	2^{NS}	2^{NS}
	0.6	59	0^{NS}	5^{NS}	5^{NS}
	2.4	60	5^{NS}	10**	20**
UV-A	0.2	60	2^{NS}	10**	12*
	0.6	60	5^{NS}	7*	10^{NS}
	2.4	60	10**	17***	17**

[a]Number of individuals. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: not significant ($P > 0.05$) between the control and UV treatments within the same column by Fisher's exact test, and DD: continuous darkness.

In diapausing females, significant differences were detected between the escape rates in all the UV treatments and those under DD even on day 1 ($P < 0.001$), and almost half of the diapausing females under UV irradiation escaped from the leaf disks on day 3 (Table 6.5).

Table 6.5 Daily changes in the escape rate in diapausing *Tetranychus urticae* adult females under continuous darkness and UV radiation.

Treatment	Irradiance [W m^{-2}]	N^a	Cumulative escape rate [%]		
			Day 1	Day 2	Day 3
Control (DD)	—	72	3	8	10
UV-C	0.06	52	19***	35***	38**
	0.2	57	21***	35***	40***
	0.6	65	14***	42***	51***
UV-B	0.2	50	38***	68***	68***
	0.6	52	27***	42***	44***
	2.4	60	28***	52***	63***
UV-A	0.2	50	32***	50***	50***
	0.6	50	40***	66***	72***
	2.4	59	42***	63***	64***

[a]Number of individuals. ***: $P < 0.001$ between the control and UV treatments within the same column by Fisher's exact test, DD: continuous darkness.

Oviposition

The oviposition rates on day 1 were significantly higher under UV-C ($P < 0.05$, Dunnett's test) and UV-B ($P < 0.01$) irradiance of < 0.2 W m^{-2} than under DD (Table 6.6). Contrary to this result, even when the UV-C and UV-B irradiances were as low as 0.06 W m^{-2} and 0.2 W m^{-2}, respectively, the oviposition rates on days 2 and 3 were significantly lower that those under DD ($P < 0.001$ and $P < 0.05$, respectively).

Interestingly, such inhibitory effects were not observed in the case of UV-A, and even when the irradiance was as high as 2.4 W m^{-2}, the oviposition rate was not significantly different from that under DD on day 3.

Table 6.6 Daily changes in the number of eggs laid by non-diapausing *Tetranychus urticae* adult females under continuous darkness and UV radiation.

Treatment	Irradiance [W m^{-2}]	Number of eggs laid per day					
		Day 1		Day 2		Day 3	
Control (DD)	—	1.4±0.1[a]	(97[b])	8.3±0.2	(96)	9.4±0.3	(91)
UV-C	0.06	2.0±0.1**	(60)	6.0±0.3***	(59)	5.7±0.4***	(54)
	0.2	1.9±0.2*	(64)	3.4±0.3***	(62)	2.2±0.4***	(53)
	0.6	1.8±0.1[NS]	(63)	1.9±0.2***	(51)	0.6±0.2***	(22)
UV-B	0.2	2.0±0.2**	(60)	7.2±0.2*	(58)	6.9±0.3***	(57)
	0.6	1.7±0.1[NS]	(58)	5.2±0.3***	(55)	3.6±0.3***	(55)
	2.4	1.8±0.2[NS]	(57)	3.3±0.2***	(52)	1.6±0.3***	(29)
UV-A	0.2	1.5±0.1[NS]	(59)	8.2±0.3[NS]	(54)	9.1±0.3[NS]	(53)
	0.6	1.6±0.1[NS]	(57)	8.8±0.3[NS]	(55)	9.6±0.3[NS]	(51)
	2.4	1.8±0.2[NS]	(54)	8.3±0.3[NS]	(50)	9.2±0.4[NS]	(50)

[a]Mean ± SE. [b]Number of surviving females. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, NS: not significant between the control and UV treatments within the same column by Dunnett's test, and DD: continuous darkness.

Dose-response curves

A significant positive correlation was detected between the total UV dose for 3 days and the mortality plus escape rates in non-diapausing females in the UV-C (R^2 = 0.99, P = 0.00050, correlation analysis) and UV-B (R^2 = 0.99, P = 0.041) treatments (Fig. 6.3). The median effective dose (ED_{50}) values for 50% mortality plus escape rate in non-diapausing females were 21 and 104 kJ m^{-2} for the UV-C and UV-B treatments, respectively.

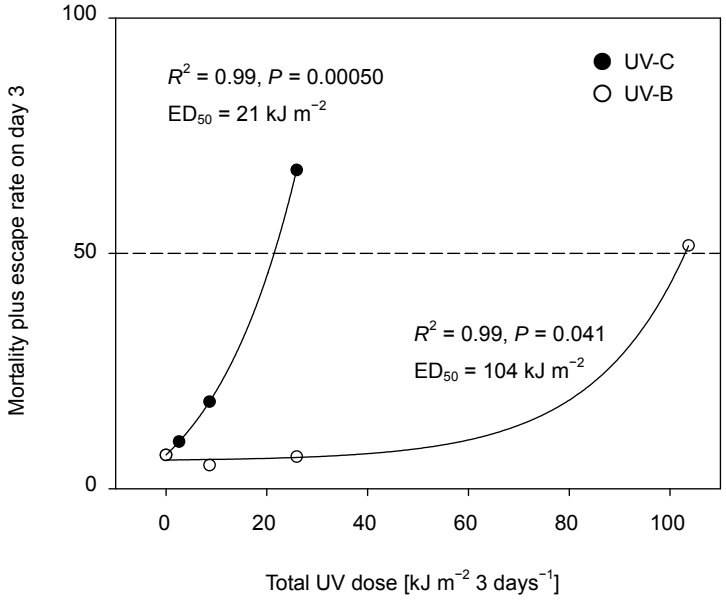

Fig. 6.3 Dose-response relationship between the total UV dose and mortality plus escape rate in non-diapausing *Tetranychus urticae* adult females.

A significant positive correlation was detected between the total UV dose for 3 days and the oviposition rates in the UV-C (R^2 = 0.99, P = 0.0021) and UV-B (R^2 = 0.97, P = 0.015) treatments (Fig. 6.4). The ED_{50} values for 50% suppression of the oviposition rate under DD (9.6 eggs for 3 days) were 6.2 and 41 kJ m^{-2} for the UV-C and UV-B treatments, respectively.

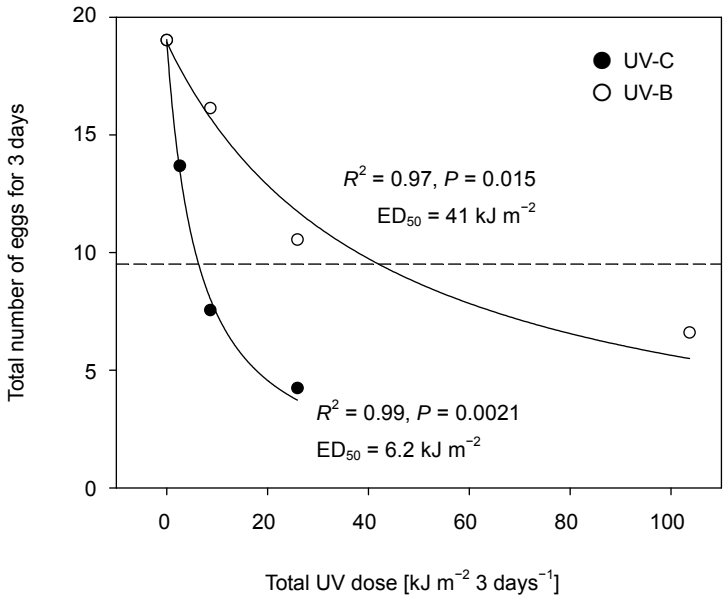

Fig. 6.4 Dose-response relationship between the total UV dose and number of eggs laid by non-diapausing *Tetranychus urticae* adult females.

Discussion

UV-B directly damages DNA and is a strong oxidative stressor because it induces the homolysis of hydrogen peroxide (H_2O_2) into hydroxyl radical ($^{\bullet}OH$), which seems to be the most damaging of the free radicals (Tan et al., 2007). UV-B-induced damage would be lethal to small organisms such as mites. In my experiments with the mite *T. urticae*, I observed that UV-B promoted the mortality (Table 6.2) and escape (Table 6.4) and inhibited oviposition (Table 6.6) in non-diapausing adult females. Moreover, these effects of UV-C were stronger than those of UV-B; the ED_{50} values for 50% mortality plus escape rate and 50% suppression of the oviposition rate under DD were 21 and 6.2 kJ m^{-2} for the UV-C treatment and 104 and 41 kJ m^{-2} for the UV-B treatment, respectively (Figs. 6.3, 6.4). These values for the UV-B treatment are actually observed in the field for 2–5 days during summer (Fig. 6.5). Therefore, the UV-B constituting solar radiation must be critical for survival and oviposition in non-diapausing females.

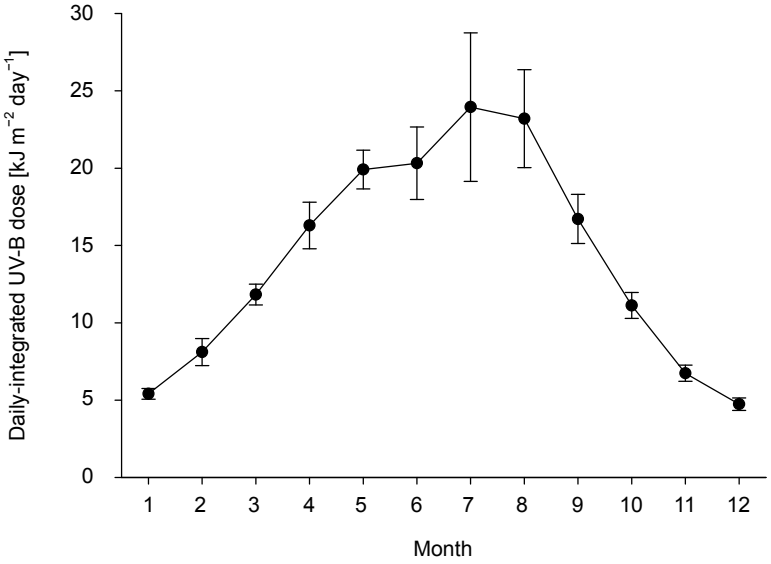

Fig. 6.5 Seasonal changes in the daily integrated UV-B radiation at Tsukuba, Japan (36°03′N). Data are expressed as the mean of the values from 1997 to 2007, and they were obtained from the Japan Meteorological Agency. Vertical bars indicate ± SD.

Mites inhabit the underside of leaves with dense vegetation in summer when they are in the non-diapause (reproductive) state. Most UV radiation is absorbed and reflected by leaves (Fig. 6.6). Therefore, the underside of leaves is considered to be a suitable environment for mites when they need to avoid UV radiation, particularly UV-B (UV-C is completely absorbed by the ozone layer). Thus, they can safely go through their reproductive cycle on the underside of leaves. However, in autumn, leaves start turning yellow and red as winter approaches and finally fall. During this phenological event, the UV-B level in the plant canopy would increase dramatically while female mites enter diapause as their body color changes from yellow-green to orange.

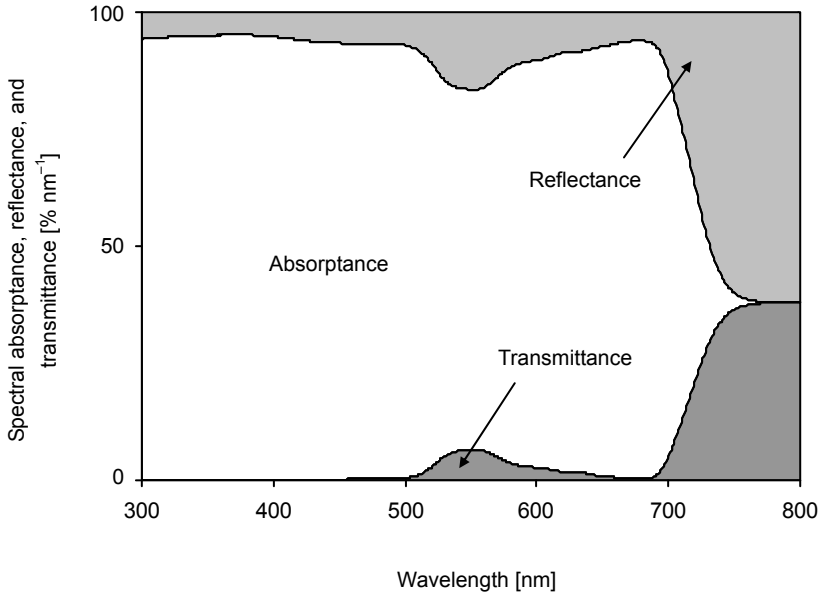

Fig. 6.6 Spectral characteristics of the kidney bean leaf. Spectral reflectance and transmittance for the bean leaf were measured with a spectrophotometer equipped with an integrating sphere (ISV-469; JASCO Co., Tokyo, Japan), and the absorptance of the leaf was calculated. For measurements, I used the first true leaves of the kidney bean plants 14 days after germination.

In diapausing females, UV-C and UV-B did not result in an increase in the mortality rates (Table 6.3), while UV-C, UV-B, and also UV-A irradiation, were observed to highly increase the escape rate (Table 6.5). It is known that diapausing females show negative phototaxis (Bondarenko, 1958; Hussey and Parr, 1963; Parr and Hussey, 1966; McEnroe, 1971). The best way to avoid the deleterious effects of UV radiation is by migrating to UV-free environments. Therefore, the strong negative phototaxis observed in diapausing females can be an adaptation to survive for several months in the absence of leaves as UV-cut filters. However, the reason why the females could respond to UV-C, which does not reach the earth's surface, remains unknown. It is known that the orange color is attributable to the accumulation of carotenoids (Veerman, 1974), which acts as a scavenger for ROS (Rousseau et al., 1992). Therefore, the low mortality rates observed in diapausing females under UV-C and UV-B irradiation may be a result of carotenoid accumulation or merely an increase in the escape rate. Therefore, it is still uncertain as to whether the diapausing females who escaped were resistant to UV-C and UV-B damage. Recently, Suzuki et al. (2008b) reported that melatonin (*N*-acetyl-5-methoxytryptamine) and its synthetic enzyme arylalkylamine *N*-acetyltransferase (NAT, EC 2.3.1.87) in non-diapausing females of *T. urticae* were activated by exposure to high-dose UV-B. It has been known that melatonin functions as an ROS scavenger (Reiter et al., 2000). Therefore, the melatonin response might also be related to the difference in UV resistance between non-diapausing and diapausing females.

In conclusion, my findings suggest that UV-B is an effective non-chemical measure for the reduction of the mite population and that the selection of habitat and change in body color is a strategy adopted by mites to reduce the deleterious effects of UV-B.

Chapter 7

UV radiation elevates arylalkylamine *N*-acetyltransferase activity in the two-spotted spider mite, *Tetranychus urticae*

Abstract

Ultraviolet (UV) radiation produces reactive oxygen species (ROS) in mammals, where melatonin plays the role of a ROS scavenger. The melatonin synthetic enzyme arylalkylamine *N*-acetyltransferase (NAT) is a significant element in a possible ROS removal system. Changes in NAT activity and melatonin content were determined in the two-spotted spider mite *Tetranychus urticae* by irradiating it with monochromatic light using the Okazaki Large Spectrograph at the National Institute for Basic Biology, Okazaki, Japan. The NAT activity and melatonin content were suppressed by blue light (450 nm). No effects of red light (650 nm) on the NAT activity and melatonin content were observed. UV radiation had intensity-dependent dual effects on the NAT activity and melatonin content. In the UV-B (300 nm) treatment, the NAT activity and melatonin content were suppressed at the intensity below 1 μmol m^{-2} s^{-1} but elevated when the intensity was as high as 10 μmol m^{-2} s^{-1}. In the UV-A (350 nm) treatment, the melatonin content was elevated when the intensity was as high as 10 μmol m^{-2} s^{-1}, though the NAT activity and melatonin content were suppressed at the intensity below 10 μmol m^{-2} s^{-1} and 1 μmol m^{-2} s^{-1}, respectively. Elevation of the NAT activity and melatonin content by high intensity UV irradiation may indicate that the UV signals initiate melatonin synthesis for ROS removal in mites.

Introduction

Ultraviolet (UV) radiation is absorbed by some coenzymes and pigments in vivo, raising them to an excited-state; the excitation energy is finally transferred to H_2O molecules yielding reactive oxygen species (ROS). It has been suggested that the UV-induced formation of ROS can induce damage to the DNA (Beehler et al., 1992) and activate the transcriptional factor "activating protein (AP)-1" (Devary et al., 1992). In mammals, AP-1 seems to promote the transcription of arylalkylamine *N*-acetyltransferase (NAT, EC 2.3.1.87) (Estrada-Rodgers et al., 1998) and 5-hydroxyindole-*O*-methyltransferase (HIOMT, EC 2.1.1.4) (Rodriguez et al., 1994), which are melatonin (*N*-acetyl-5-methoxytryptamine) synthetic enzymes. Melatonin functions as a ROS scavenger (Reiter et al., 2000). Therefore, AP-1 may transmit UV signals to the system for melatonin synthesis to remove ROS. Actually, the melatonin concentration in the roots of the plant *Glycyrrhiza uralensis* is increased by UV-B irradiation (Afreen et al., 2006). The ROS removal system of melatonin might be an evolutionarily conserved utility common to animals and plants.

The two-spotted spider mite, *Tetranychus urticae* is distributed worldwide and is a serious pest for a wide variety of crops (van de Vrie et al., 1972). In *T. urticae*, the response to photoperiod has been well investigated. Only adult females enter diapause to survive during the winter by sensing long-night conditions (Veerman, 1985). Although abundant data about photoperiodism has been accumulated, other knowledge on the response to light remains limited. Here, I report the NAT activity and melatonin content in *T. urticae* by employing a radio enzymatic assay and radioimmunoassay, respectively. These sensitive assays enable the monitoring of the ROS removal system by melatonin. In this study, I investigated a relationship between the UV radiation and

melatonin synthesis in *T. urticae*.

Materials and methods

Laboratory culture of T. urticae

The founder population of *T. urticae* was collected from an apple (*Malus pumila* Mill. cv. Fuji) orchard at Akita, Japan (39°15′N), in 2001. The offspring populations were maintained in the laboratory on kidney bean leaves (*Phaseolus vulgaris* L.) under long-day (LD 16:8) conditions provided by white fluorescent lamps at 25°C. A total of 150 adult females emerged from the teleiochrysalis stage within 2 d; they were then placed onto a fresh bean leaf kept over water-soaked cotton in a plastic petri dish (diameter, 9 cm; depth, 2 cm).

Monochromatic light irradiation

To determine the effects of the spectral quality of light on the NAT activity and melatonin content, *T. urticae* was exposed under the Okazaki Large Spectrograph (OLS) (Watanabe et al., 1982) at the National Institute for Basic Biology, Okazaki, Japan. Monochromatic light was provided by a large spectrograph equipped with a 30-kW xenon arc lamp (Ushio Electric Co., Tokyo, Japan). The light beam was first reflected by a plane mirror and then by a condensing mirror. After reflection by a diffraction grating, the light beam passed into the irradiation room. In each petri dish placed at various wavelengths of 300, 350, 450 and 650 nm, *T. urticae* samples were placed beneath a mirror that reflected the monochromatic light beam directly onto them. Table 7.1 lists the treatment codes, wavelength peaks, and irradiance when the photon flux was set at 10 μmol m^{-2} s^{-1}. Figure 7.1 shows the spectral distributions of the

treatments when the photon flux was set at 100 μmol m^{-2} s^{-1}. Short-cutoff filters (Hoya Co., Tokyo, Japan) were placed above the petri dishes. The photon fluxes examined (0.01–10 μmol m^{-2} s^{-1} and 0.1–10 μmol m^{-2} s^{-1} for NAT and melatonin assay, respectively) at specific wavelengths were adjusted by means of neutral-density filters (Fujitok Co., Tokyo, Japan) and measured using a photon-flux density meter (QTM-101, Monotech Inc., Saitama, Japan). Samples were treated in a light–dark cycle (LD 4:20) under monochromatic lights with different fluence rates. Samples for the NAT and melatonin assays were frozen after the third and forth exposure intervals, respectively, and stored at −80°C.

Table 7.1 Wavelength peak and irradiance for the photon flux of 10 μmol m^{-2} s^{-1}

Treatment code	Wavelength peak [nm]	Irradiance [W m^{-2}]
UV-B	300	3.85
UV-A	350	3.17
Blue	450	2.50
Red	650	1.78

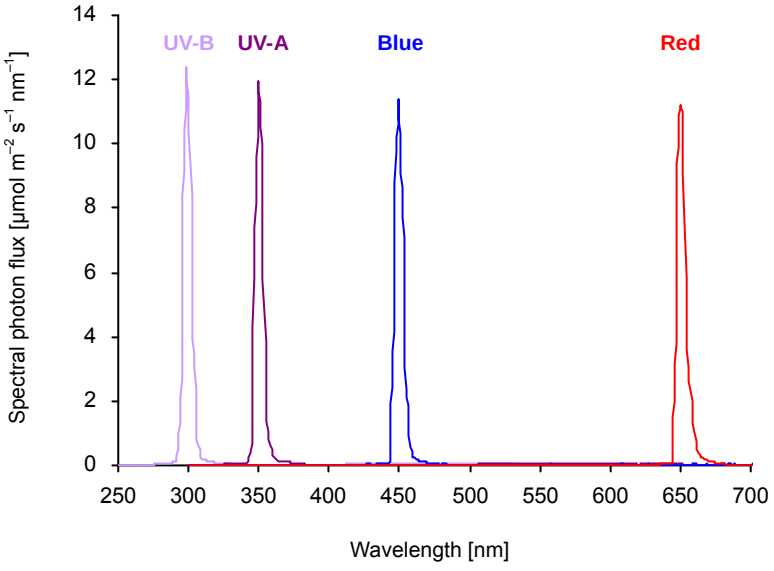

Fig. 7.1 Spectral distributions of the treatments when the photon flux was set at 100 μmol m^{-2} s^{-1}.

NAT activity assay

The NAT activity assay was performed according to Deguchi and Axelrod (1972) and Ichihara et al. (2001) with some modifications. Samples (10 mites/tube) were homogenized by sonication in a homogenization solution {pH 7.4; 20 mM HEPES, 20 mM NaOH, 1 mM ethylenediaminetetraacetic acid (EDTA)-2Na, 1 mM dithiothreitol (DTT), 100 μg mL^{-1} phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, 0.01% Nonidet P-40, 10 μg mL^{-1} aprotinin, 10 μg mL^{-1} leupeptin, and 1 μg mL^{-1} pepstatin A}. The homogenized samples were centrifuged at 8000 × *g* for 15 min at 4°C. The supernatant was used as a crude enzyme solution. In the standard procedure, the reaction mixture consisted of 2 μL of the crude enzyme solution, 2 μL of 5-HT as the substrate, 2 μL of 0.4 mM acetyl-CoA containing 0.1 mM [^{14}C] acetyl-CoA (specific activity, 2.0 TBq mol^{-1}; Moravek Biochemicals, Brea, CA, USA) and 0.3 mM cold acetyl-CoA (hot/cold = 1:3), and 14 μL of Clark and Lubs solution (pH 7.6) (Clark and Lubs, 1917) in a final volume of 20 μL in a polypropylene tube. After incubation at 37°C for 2 min, the reaction was terminated by the addition of 100 μL of 5% acetic acid. A liquid scintillation cocktail {(volume, 1 mL) (4 g L^{-1} 2,5-diphenyloxazole (PPO) and 0.1 g L^{-1} 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP) in toluene/isoamyl alcohol (7:3 v/v)} was added to the reaction mixture. The mixture was vortexed for 30 s and centrifuged at 800 × *g* for 3 min. The radioactivity in the organic layer was measured using a liquid scintillation counter (LSC-700; Aloka Co. Ltd., Tokyo, Japan). The NAT activity was estimated by subtracting the activity of the minus-enzyme control from the measured activity.

Radioimmunoassay for melatonin

To measure melatonin content, the samples (10 mites/tube) were homogenized by sonication in 100 μL chloroform. The extract was dried overnight at 4°C. Five hundred microliters assay buffer was added to dissolve the dried content. The assay buffer was tricine-gelatin buffer, consisting of 0.9% NaCl, 1.8% tricine and 0.1% gelatin, adjusted to pH 7.5. Radioimmunoassay for melatonin was performed according to Fraser et al. (1983) and Bembenek et al. (2005) with some modifications. The anti-melatonin serum and [^{3}H]-melatonin were purchased from Stockgrand (Guildford, Surrey, UK) and GE Healthcare Bio-Sciences (Tokyo, Japan), respectively. [^{3}H]-Melatonin had a specific activity of 9.25 MBq. Crystalline melatonin was obtained from Sigma (St. Louis, MO), and a stock solution of 1 mg mL^{-1} melatonin standard was prepared. The stock solution was further diluted with assay buffer to give individual standards ranging from 0.8 to 100 pg 100 μL^{-1}. The antiserum was stored at −80°C and diluted with assay buffer before use to give as initial dilution of 1:600. Two hundred microliters sample (duplicate determinations) were added to a tube containing an additional 100 μL assay buffer, 100 μL [^{3}H] melatonin and 100 μL diluted antibody. After incubation for 24 h at 4°C, 500 μL of DCC (Dextran Coated Charcoal; 0.27 mg mL^{-1}) were added to the reaction mixture, which was left to stand for 15 min at 4°C. This mixture was centrifuged at 700 × *g* for 15 min at 4°C and the supernatant was added to vials containing 3.6 mL scintillation cocktail (Atomlight, Perkin-Elmer Inc., Wellesley, MA, USA). The radioactivity in the vial was measured using the liquid scintillation counter.

Protein measurement

The protein content was determined according to Bradford (1976) by using bovine serum albumin as the standard (B4287; Sigma). The values are provided as the mean of duplicate determination.

Data analysis

For the data (NAT activity and melatonin content), the mean values and standard errors were calculated from 3 to 5 replicates. The statistical significance of the differences between the data under continuous darkness (DD) and those changed by the exposure to different photon fluxes at a specific wavelength was tested by a one-way analysis of variance (ANOVA), followed by Dunnett's test (Dunnett, 1955). The statistical significance of the differences in the data among the different wavelengths at a specific photon flux was tested by one-way ANOVA, followed by Tukey's test. Calculations were performed using the statistical package for the social sciences (SPSS) 11.5J software (SPSS Japan Inc., Tokyo, Japan).

Results

Quantum effect

In the UV-B treatment, the difference between the NAT activity at the photon flux of 0.01 μmol m^{-2} s^{-1} and that obtained under DD was not significant ($P > 0.05$) (Fig. 7.2a). However, at photon fluxes of 0.1 and 1 μmol m^{-2} s^{-1}, the NAT activities decreased after the abovementioned treatment and were significantly lower than those obtained under DD ($P < 0.01$). Contrary to the results obtained at the photon flux

below 1 μmol m^{-2} s^{-1}, the NAT activity increased by UV-B at the photon flux of 10 μmol m^{-2} s^{-1} and was significantly higher than that obtained under DD ($P < 0.05$).

The NAT activities after the exposure were significantly lower than those obtained under DD even when the photon flux was as low as 0.01 μmol m^{-2} s^{-1} in the UV-A ($P < 0.001$) and blue ($P < 0.05$) treatments (Figs. 7.2b, c).

In the red treatment, the differences between the NAT activities and those obtained under DD were not significant ($P > 0.05$) even if the photon flux was as high as 10 μmol m^{-2} s^{-1} (Fig. 7.2d).

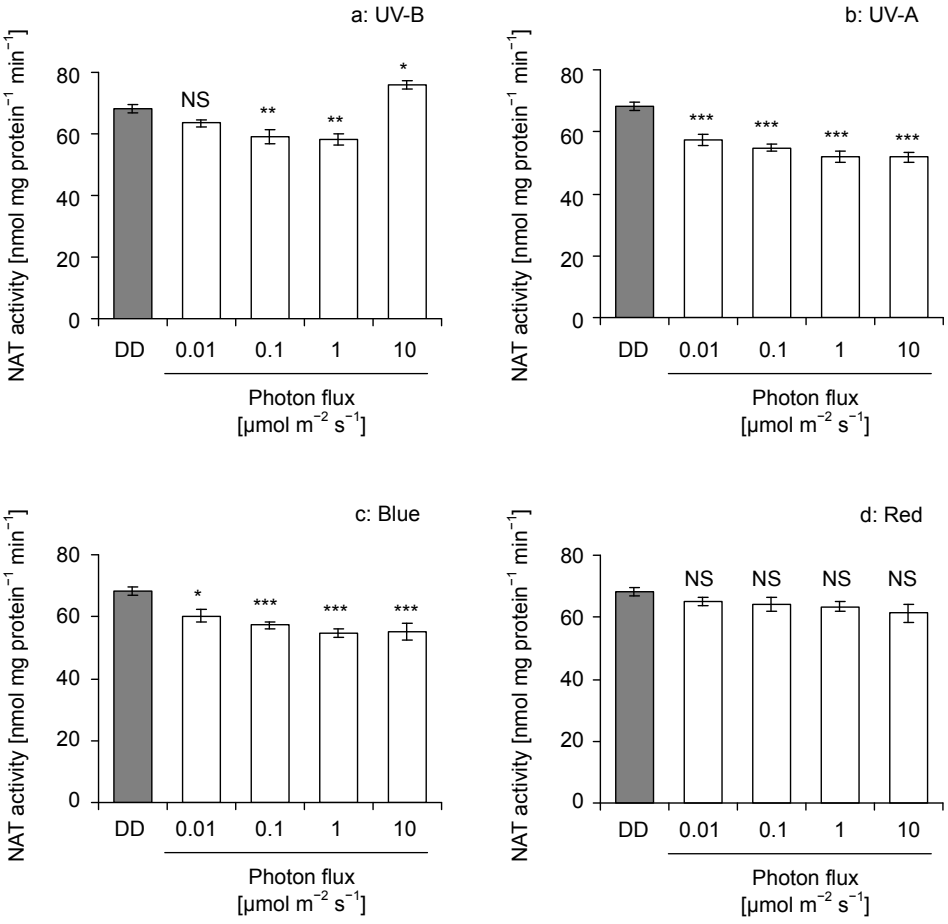

Fig. 7.2 Comparison of the NAT activity in *Tetranychus urticae* under DD (continuous darkness) with that under each photon flux for a: UV-B, b: UV-A, c: blue, and d: red light treatments. The samples were kept under DD and exposed to monochromatic lights with different photon fluxes for 3 d (4 h d^{-1}). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, and NS: not significant by Dunnett's test. Vertical bars indicate ±SE of 3–5 experiments; $N = 10$.

In the UV-B and -A treatments, the melatonin contents after the exposure were significantly lower than those obtained under DD, when the photon flux was 0.1 (P < 0.01) and 1 (P < 0.001) μmol m^{-2} s^{-1} (Figs. 7.3a, b). Contrary to the results obtained at the photon flux below 1 μmol m^{-2} s^{-1}, the melatonin contents increased by UV-B and -A at the photon flux of 10 μmol m^{-2} s^{-1}. However, no significant differences were detected between the melatonin contents in UV-B and -A treatments at the photon flux of 10 μmol m^{-2} s^{-1} and those obtained under DD (P > 0.05).

In the blue treatment, the melatonin content after the exposure decreased and were significantly lower than that obtained under DD, when the photon flux was 0.1 (P < 0.05), 1 and 10 (P < 0.01) μmol m^{-2} s^{-1} (Fig. 7.3c).

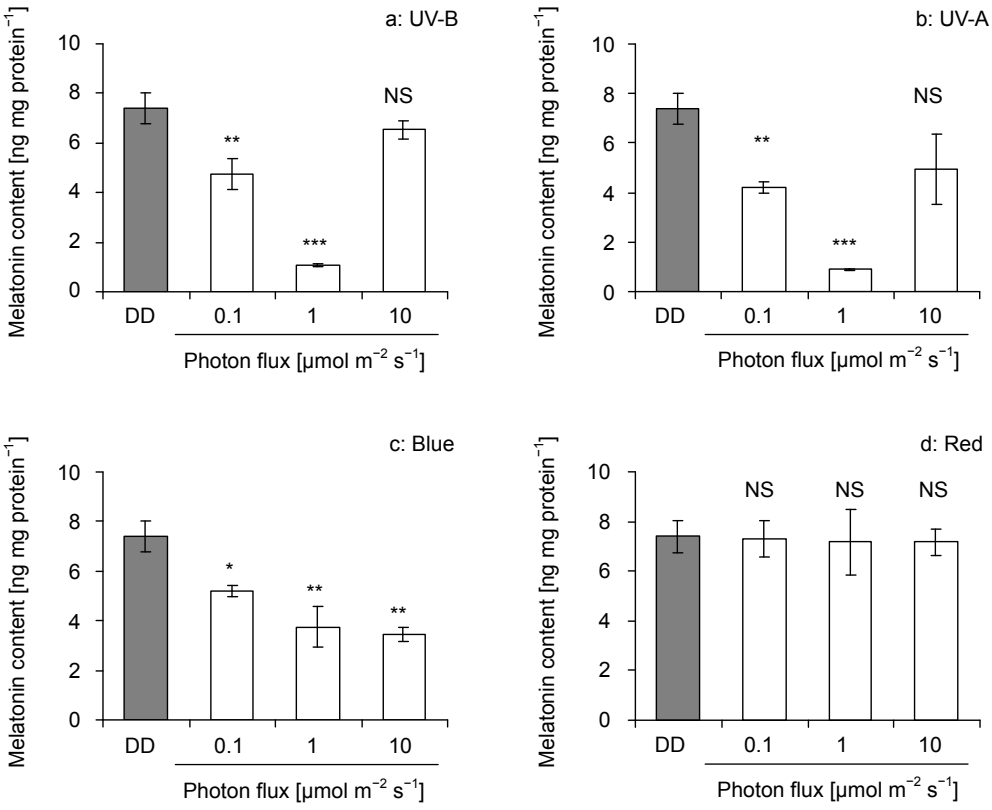

Fig. 7.3 Comparison of the melatonin content in *Tetranychus urticae* under DD with that under each photon flux for a: UV-B, b: UV-A, c: blue, and d: red light treatments. The samples were kept under DD and exposed to monochromatic lights with different photon fluxes for 4 d (4 h d^{-1}). *: P < 0.05, **: P < 0.01, ***: P < 0.001, and NS: not significant by Dunnett's test. Vertical bars indicate ±SE of 3–5 experiments; N = 10.

For the red treatment, the differences between the melatonin contents and those obtained under DD were not significant ($P > 0.05$) even if the photon flux was as high as 10 μmol m^{-2} s^{-1} (Fig. 7.3d).

Equal quantum action spectrum

The equal quantum action spectra for suppression of NAT activity at photon fluxes of 0.01, 0.1, 1, and 10 μmol m^{-2} s^{-1} show a maximum at 650 nm (red), except at 10 μmol m^{-2} s^{-1}, and a minimum at 350 nm (UV-A) (Figs. 7.4a–d). At a photon flux of 10 μmol m^{-2} s^{-1}, the maximum was obtained at 300 nm (UV-B). In all the photon fluxes, the differences between the NAT activities at 350 nm and those at 650 nm were significant ($P < 0.05$). Moreover, at the photon flux of 10 μmol m^{-2} s^{-1}, the difference between the NAT activities at 350 nm and 300 nm was significant ($P < 0.05$).

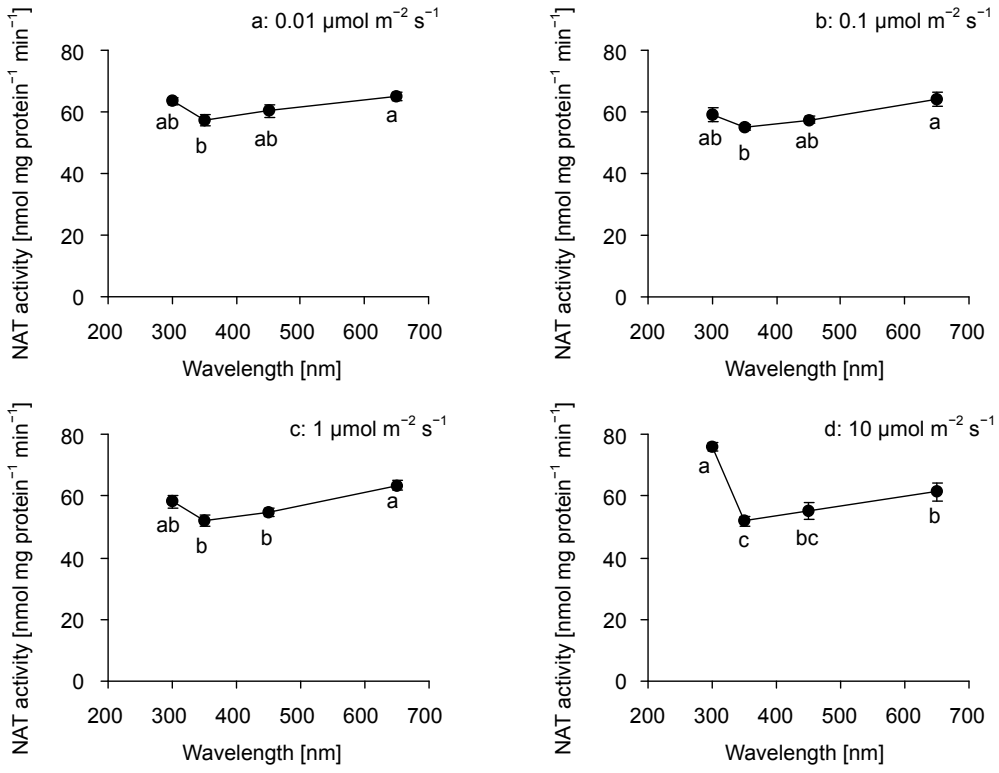

Fig. 7.4 Equal quantum action spectra for the suppression of NAT activity in *Tetranychus urticae* exposed to a: 0.01 μmol m^{-2} s^{-1}, b: 0.1 μmol m^{-2} s^{-1}, c: 1 μmol m^{-2} s^{-1}, and d: 10 μmol m^{-2} s^{-1} monochromatic lights for 3 d (4 h d^{-1}). Different letters indicate a significant difference at $P < 0.05$ by Tukey's test. Vertical bars indicate ±SE of 3–5 experiments; $N = 10$.

The equal quantum action spectra for suppression of melatonin content at photon fluxes of 0.1, 1 and 10 μmol m^{-2} s^{-1} show a maximum at 650 nm, and a minimum at 350 nm except at 10 μmol m^{-2} s^{-1} (Figs. 7.5a–c). At a photon flux of 10 μmol m^{-2} s^{-1}, the minimum was obtained at 450 nm (blue). The differences between the melatonin contents at 300 and 350 nm and those at 650 nm were not significant ($P > 0.05$) at photon flux of 10 μmol m^{-2} s^{-1}, but were significant ($P < 0.05$) at photon fluxes of 0.1 and 1 μmol m^{-2} s^{-1}. At the photon flux of 10 μmol m^{-2} s^{-1}, the difference between the NAT activities at 450 nm and 650 nm was significant ($P < 0.05$).

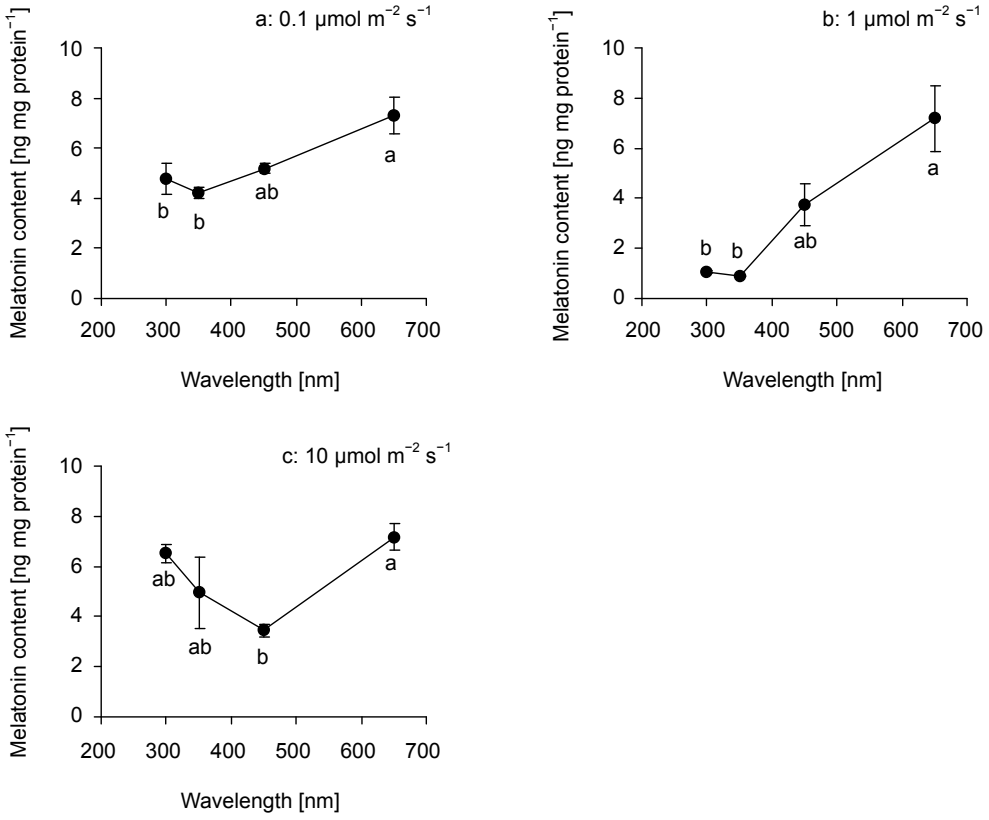

Fig. 7.5 Equal quantum action spectra for the suppression of melatonin content in *Tetranychus urticae* exposed to a: 0.1 μmol m^{-2} s^{-1}, b: 1 μmol m^{-2} s^{-1}, and c: 10 μmol m^{-2} s^{-1} monochromatic lights for 4 d (4 h d^{-1}). Different letters indicate a significant difference at $P < 0.05$ by Tukey's test. Vertical bars indicate ±SE of 3–5 experiments; $N = 10$.

Discussion

Melatonin is a substance produced by the pineal gland (Axelrod, 1974), bone marrow (Tan et al., 1999), gastral mucosa (Bubenik, 2002), lymphocytes (Carrillo-Vico et al., 2004), and even by plants (Reiter and Tan, 2002; Afreen et al., 2004), bacteria (Manchester et al., 1995), insects (Itoh et al., 1995; Hintermann et al., 1996; Chen et al., 2003; Bembenek et al., 2005), and Chelicerata (Tilden et al., 2001). In the past, melatonin was discovered to be a free radical scavenger (Tan et al., 1993; 2000; 2007) and antioxidant (Tan et al., 2000; 2007; Reiter et al., 2000) in addition to its involvement in circadian and photoperiodic systems. Further, in insects, melatonin is an efficient scavenger of hydroxyl radicals ($^{\bullet}OH$) (Anisimov et al., 1997).

UV-B is a strong oxidative stressor because it induces the homolysis of hydrogen peroxide (H_2O_2) into $^{\bullet}OH$, which seems to be the most damaging among the free radicals (Tan et al., 2007). The melatonin concentration in the roots of *G. uralensis* is increased by UV-B irradiation (Afreen et al., 2006). These suggest that the increase in the melatonin concentration was a response to the UV-B stress and that melatonin plays the role of antioxidant defense. Actually, melatonin suppresses UV-induced ROS in leukocytes (Fischer et al., 2004).

The gene expression of the melatonin synthetic enzyme NAT was raised by pancreatic oxidative stress (Jaworek et al., 2003). Moreover, under forced swimming stress, the NAT activity in the pineal was doubled as compared to that in control rats (Troiani et al., 1988). These indicate that the oxidative stress also elevates the NAT levels.

I used mites to investigate the influence of UV irradiation on the NAT activity and melatonin content. The UV-induced damage would be lethal to mites because of their

small size. Therefore, it is considered that a protection system against UV-induced free radicals has been developed in mites. However, no report is available on the investigation of the ROS removal system in mites.

In this study, the NAT activity and melatonin content in *T. urticae* were suppressed by UV-B at low intensity, UV-A, and blue light. These suppressions by light are comparable to those of the circadian responses not only in vertebrates but also in the silkworm (*Bombyx mori*) (Itoh et al., 1995) and American cockroach (*Periplaneta americana*) (Bembenek et al., 2005). In many vertebrate systems, the output signals of the biological clocks are transduced to the synthesis and release of melatonin. NAT activity exhibits circadian rhythm in the vertebrate pineal gland, which regulates the level of melatonin (Ganguly et al., 2002). In rats, circadian clock-related proteins CLOCK/BMAL1 heterodimers bind to E-box elements in the promoter of NAT gene and activate its expression (Chen and Baler, 2000). A similar mechanism of NAT expression is found in chickens (Chong et al., 2000). Moreover, oscillation of the CLOCK/BMAL1 heterodimer activity expected from circadian expression of Per1 and Per2 genes (Shearman et al., 1997) is entirely consistent with the dynamics of the nocturnal increase in NAT mRNA (Niki et al., 1998; Sakamoto and Ishida, 1998). Therefore, Chen and Baler (2000) characterize the NAT gene as a possible direct transcriptional target of the biological clock loop in a maser oscillator. In *B. mori*, distributions of immunoreactivities of CYCLE (CYC, a homolog of BMAL1 in insects) almost match that of NAT (Sehadová et al., 2004). This suggests a possibility that transcription of NAT is regulated by CLOCK/CYC in insects. Therefore, the suppressed NAT activity and melatonin content observed in the present study might be the output signal from the biological clock even in *T. urticae*.

In contrast to abovementioned results, the NAT activity and melatonin content were elevated by UV-B at high intensity. The elevated NAT activity and melatonin content seem to be a response to oxidative stress. Moreover, the melatonin content was elevated by UV-A at high intensity. However, no elevation of the NAT activity by UV-A was observed even when the intensity was high. This might be due to the difference in exposure period between the sample for the NAT assay (3 days) and that for the melatonin assay (4 days). Further investigations are required to clarify the accumulated effects of UV radiation on the NAT activity and melatonin content. In insects and mites, this is the first report describing the UV-induced NAT activity and melatonin content. Since AP-1 seems to promote the transcription of NAT (Estrada-Rodgers et al., 1998), even in *T. urticae*, AP-1 might be present in the upstream of NAT.

In conclusion, the present study suggests the presence of a ROS removal system by melatonin in *T. urticae*. Further studies to investigate the effects of UV radiation on AP-1 level and to analyze the antioxidative function of melatonin are required.

Chapter 8

Pharmacological approach to bifenazate mode of action: effects of bifenazate and its principal active metabolite, diazene, on the hindgut contraction of *Leucophaea maderae*

Abstract

To approach the mode of actions of bifenazate and its principal active metabolite, diazene, the effects of these compounds were investigated on the hindgut contraction of the cockroach *L. maderae*. The intensity and rhythm of the hindgut contraction were quantified by two parameters: amplitude and ferequency, respectively. Bifenazate and diazene possessed inhibitory effects on contraction intensity and rhythm in the hindgut; the effects were dose-depended. A comparison of EC_{50} values showed that the inhibitory effects of bifenazate were higher than those of diazene. It has been suggested that bifenazate apparently acts on the post-synaptic γ-aminobutyric acid (GABA) receptor (GABAR), which mediates predominantly inhibitory neurotransmission and is involved in spontaneous mechanical activities of visceral nerves and muscles. The inhibitory effects of bifenazate and diazene were blocked by the GABAR antagonist, bicuculline methiodide. This suggests that bifenazate and diazene stimulate the GABAR function, supporting the GABAR hypothesis for the bifenazate mode of action.

Introduction

Bifenazate is a novel carbazate acaricide discovered by Uniroyal Chemical Company Inc. (now Chemtura Corporation), Middlebury, CT, and is one of the most effective compounds for the control of spider mites of the genus *Tetranychus* and *Panonychus* (Dekeyser and McDonald, 1994; Dekeyser et al., 1994, 1996; Canlas, 2005; Ochiai et al., 2007). Currently, a commercial product containing bifenazate has been released onto the market in Japan under the trade name of Mitekohne® by Nissan Chemical Industries, Ltd., Tokyo, Japan. Its registration has been granted in many other countries (e.g. Acramite® and Floramite® trade names in USA) for spider mite control on both agricultural crops and ornamental plants (Ochiai et al., 2007).

According to Dekeyser et al. (1996), bifenazate possesses low toxicity to mammals and aquatic organisms, and breaks down quickly in the environment; bifenazate exerts rapid poisoning, no cross-resistance to many conventional acaricides and gives season-long control of the two-spotted spider mite, *T. urticae* and the citrus red mite, *Panonychus citri*; it has a high margin of safety to predatory mites and other beneficial arthropods. Actually, it was revealed that bifenazate was less toxic to the predatory mites, *Amblyseius* (*Neoseiulus*) *womersleyi* (Kim and Seo, 2001; Amano et al., 2004), *Phytoseiulus persimilis* (Kim and Yoo, 2002; Ochiai et al., 2007), and *Neoseiulus californicus* (Amano et al., 2004; Ochiai et al., 2007). Therefore, bifenazate can reconcile chemical and biological controls, i.e., it is an ideal compound for implementation of integrated pest management (IPM), as Ochiai et al. (2007) have demonstrated.

Since bifenazate shows no cross-resistance to spider mites resistant to a wide range of conventional acaricides (Grosscurt and Avella, 2005; Pree at al., 2005; Van

Leeuwen et al., 2005; Ochiai et al., 2007), its mode of action may distinct from that of other acaricides. Although no detailed investigation on the mode of action has been reported, preliminary studies suggest that bifenazate apparently acts on the post-synaptic γ-aminobutyric acid (GABA) receptor in the nervous system (Dekeyser, 2005). However, bifenazate has been classified by the Insecticide Resistance Action Committee (IRAC) in the group with unknown or uncertain mode of action (IRAC, 2008). Recently, the maternal inheritance of bifenazate resistance was demonstrated by Van Leeuwen et al. (2006, 2007, 2008) who also suggested that the mode of action might not involve a site of action in the GABA receptor (GABAR) but cytochrome *b*, a mitochondrially encoded protein in the respiratory pathway of spider mites. These authors postulated that a breakdown product of bifenazate was responsible for its activity. Diazene, the principal active metabolite of bifenazate, is also a very potent acaricide and its selectivity is paralleled that of bifenazate but its toxicity to *T. urticae* is higher than bifenazate (Canlas, 2005; Ochiai et al., 2007). However, the action mechanism of diazene is still unknown.

Insect gut has a property of peristaltic movement, which may be controlled by its own visceral nerves and muscles (Kanehisa, 1966a). Using this property, effects of cholinesters, bioactive amines, amino acids, peptide, insecticides, and temperature were investigated in the cockroach, *Periplaneta americana* (Kanehisa, 1965, 1966a, b; Penzlin, 1994; Nässel et al., 1998; Predel et al., 2001; Sakai et al., 2004) and *Leucophaea maderae* (Nässel et al., 1998). However, it is difficult to use spider mite gut because the size is too small. Therefore, in this study, I used the hindgut of *L. maderae*. Then, I pharmacologically investigated the effects of bifenazate, diazene, and a GABAR antagonist on hindgut contraction of *L. maderae* to approach to the GABAR hypothesis in the bifenazate mode of action.

Materials and Methods

Laboratory culture of L. maderae

Male and female specimens of *L. maderae* reared in mass and fed with water and artificial diet (MF; Oriental Yeast Co. Ltd., Tokyo, Japan) in continuous darkness at 25°C were used for the experiment.

Chemicals

Technical grade bifenazate and diazene were provided by Uniroyal Chemical (Chemtura). Both chemicals were dissolved in methanol. As a GABAR antagonist, bicuculline methiodide (BMI) (B6889; Sigma, St. Louis, MO) was used and dissolved in distillated water. No effects of methanol and distillated water on the hindgut contraction were previously confirmed (data not shown).

Gut contraction assay

The hindgut of *L. maderae* adult males and females was used for bioassay to confirm the effects of bifenazate and diazene. After dissection, the hindgut was mounted in a plastic chamber (2 mL) containing insect saline of the following composition as described by Sakai et al. (2004): 154 mM NaCl, 13 mM KCl, 1 mM $CaCl_2$ and 11 mM D(+)-glucose in 10 mM HEPES (pH 7.0).

To measure the contraction, both ends of the hindgut were bound by a cotton thread and one end was connected to the tension sensor (TB611-T; Nihon Kohden, Tokyo, Japan). The other end was attached to a wire fixed at the bottom of the chamber.

The chemicals were applied in 2 μL volumes to obtain the desired concentration in the chamber. Data were recorded every 0.5 s.

Data analysis

To determine effects of chemicals on the hindgut contraction, two parameters (amplitude and frequency) were used and the ratios of change in these parameters between before and after application of the chemicals were calculated.

Each 512 data before and after applying chemicals was respectively picked up for the analysis of the amplitude and frequency. The latter data was started to pick up 10 s (20 data) after the application of the chemicals to avoid drastic physical effects. The zero point compensation was performed; the minimum value within the total (1024) data was adjusted to 0.

The revised contraction data and its regression line were regarded as functions $f(t)$ and $g(t)$, respectively (t: time). Then, the area (S) enclosed by $f(t)$ and $g(t)$ was regard as an index of the amplitude (contraction intensity) and calculated by following equation (b and a indicate before and after applying chemicals, respectively):

$$S_{\mathrm{b}} = \int_{0}^{255.5} f(t) - g(t)$$

$$S_{\mathrm{a}} = \int_{266}^{521.5} f(t) - g(t)$$

$$f(t) - g(t) \geq 0$$

The frequency was determined from the power spectrum calculated by using the fast Fourier transform (FFT) algorism in the Berkeley Madonna: Modeling and Analysis of Dynamic Systems (Version 8.3.9, University of California at Berkeley,

http://www.berkeleymadonna.com). The peak frequencies in the power spectrum before and after application of the chemicals were defined as f_b and f_a to determine the effects on rhythm of the hindgut contraction.

A correlation analysis was performed to determine the dose-response curves in the ratios of change in amplitude and frequency between before and after application of the chemicals, and to calculate each pEC_{50} ($-\log EC_{50}$) value. The calculations were conducted based on the SigmaPlot 2004 software (Version 9.9, Systat Software Inc., Richmond, CA).

Results

Effects of bifenazate and diazene

The hindgut contraction intensity of *L. maderae* was inhibited by 10^{-6} M bifenazate and 10^{-7} M diazene; when the S_b values were set at 1.0, the S_a values in the above-mentioned bifenazate and diazene treatments were 0.57 and 0.90, respectively (Figs. 8.1a, c).

The peak frequency of rhythm of the hindgut contraction was reduced from f_b [0.035 Hz (28 s^{-1})] to f_a [0.023Hz (43 s^{-1})] by 10^{-6} M bifenazate and from f_b [0.031 Hz (32 s^{-1})] to f_a [0.027 Hz (37 s^{-1})] by 10^{-7} M diazene (Figs. 8.1b, d).

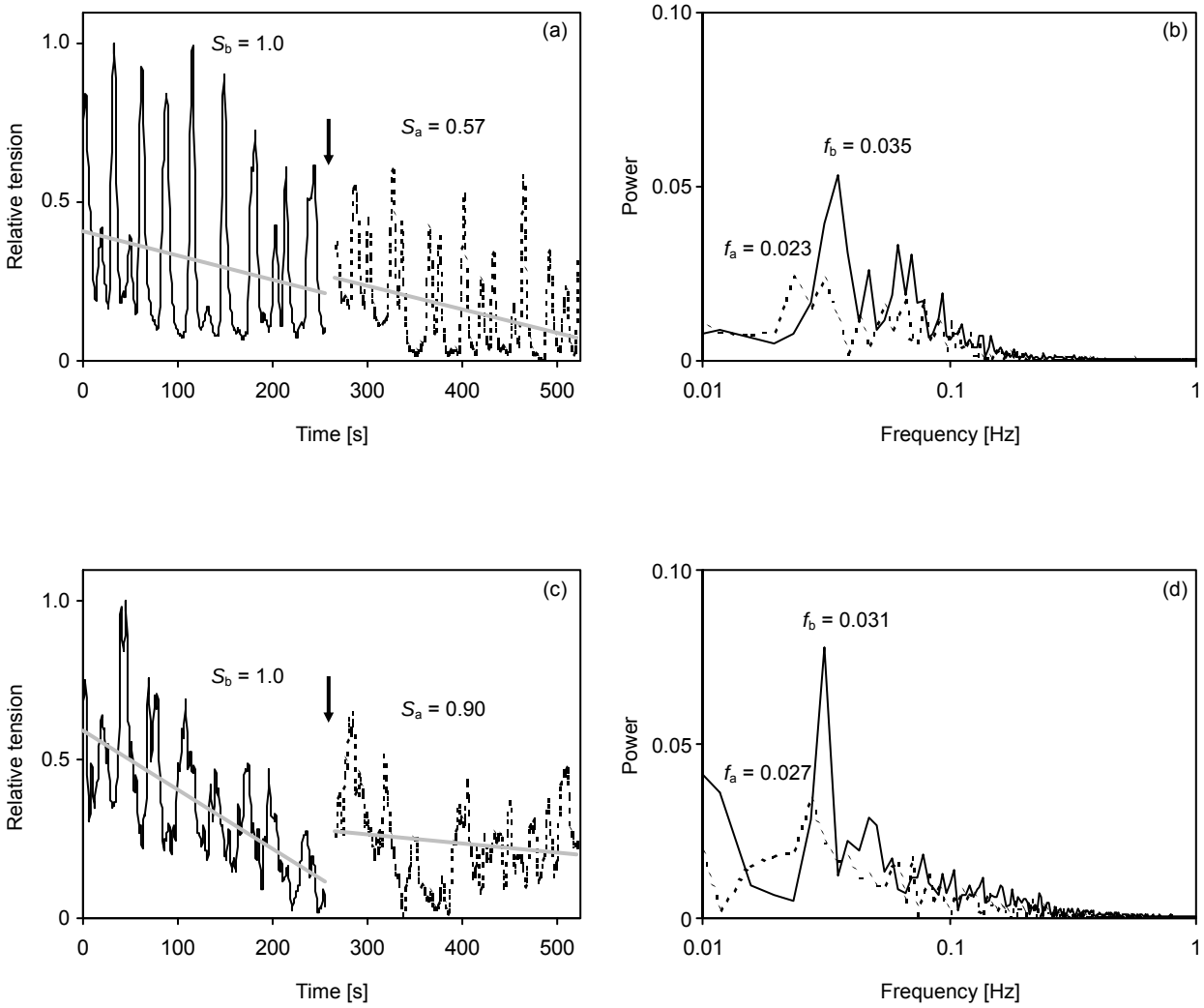

Fig. 8.1 Effects of (a) 10^{-6} M bifenazate and (c) 10^{-7} M diazene on the hindgut contraction of *Leucophaea maderae*. Power spectra (b) and (d) were calculated by the fast Fourier transform (FFT) of wave signals (a) and (c), respectively. Solid and broken lines indicate before and after applying chemicals, respectively. Arrows indicate the time of application of a chemical. The S_b and S_a indicate an index of the amplitude of the hindgut contraction before and after applying chemicals, respectively. The f_b and f_a indicate the peak frequency of the hindgut contraction before and after applying chemicals, respectively. The gray lines were calculated by a regression analysis.

Dose-response curves

A negative correlation was detected between the logarithmic concentration of bifenazate and gut contraction parameters: amplitude (S_a / S_b) and frequency proportion (f_a / f_b) (Figs. 8.2a, b). The pEC_{50} values in the amplitude and frequency proportion were 6.2 and 9.0 M, respectively. The Hill slopes in the amplitude and frequency proportion were −0.56 and −4.6, respectively.

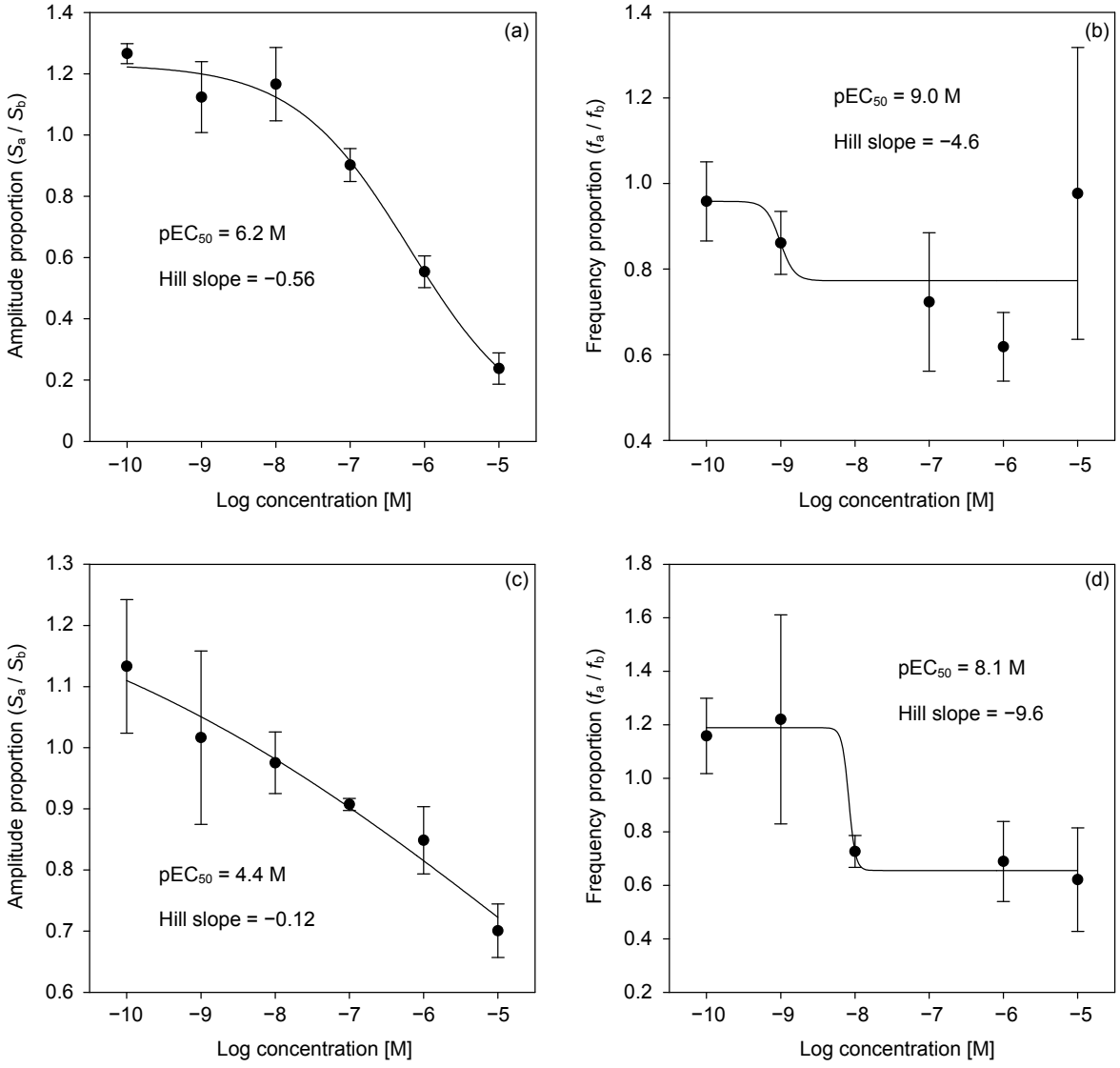

Fig. 8.2 Dose-dependent effects of bifenazate (a, b) and diazene (c, d) on the amplitude (a, c) and frequency (b, d) of the hindgut contraction of *Leucophaea maderae*. Vertical bars indicate ±SE of 3 experiments. pEC_{50} indicates $-\log EC_{50}$.

A negative correlation was also detected between the logarithmic concentration of diazene and gut contraction parameters: amplitude (S_a / S_b) and frequency proportion (f_a / f_b) (Figs. 8.2c, d). The pEC_{50} values in the amplitude and frequency proportion were 4.4 and 8.1 M, respectively. The Hill slopes in the amplitude and frequency proportion were −0.12 and −9.6, respectively.

Effects of GABAR antagonist

The inhibitory effects of bifenazate and diazene in the concentrations of 10^{-6} M on the hindgut contraction were blocked by the pretreatment with BMI at 10^{-9} M and 10^{-8} M, respectively (Fig 8.3). No reduction of the amplitude was observed; when the S_b values were set at 1.0, the S_a values in bifenazate and diazene treatments were 1.2 and 1.0, respectively (Figs. 8.3a, c).

The peak frequency of rhythm of the hindgut contraction was reduced by applying bifenazate from f_b [0.043 Hz (23 s^{-1})] to f_a [0.031Hz (32 s^{-1})] by the pretreatment with BMI (Fig 8.3b). However, the f_a / f_b value (0.72) was slightly higher than the value (0.66) observed in Figure 8.1b (without BMI pretreatment). The peak frequency in the diazene treatment slightly increased from f_b [0.039 Hz (26 s^{-1})] to f_a [0.043Hz (23 s^{-1})] by the pretreatment with BMI (Fig. 8.3d).

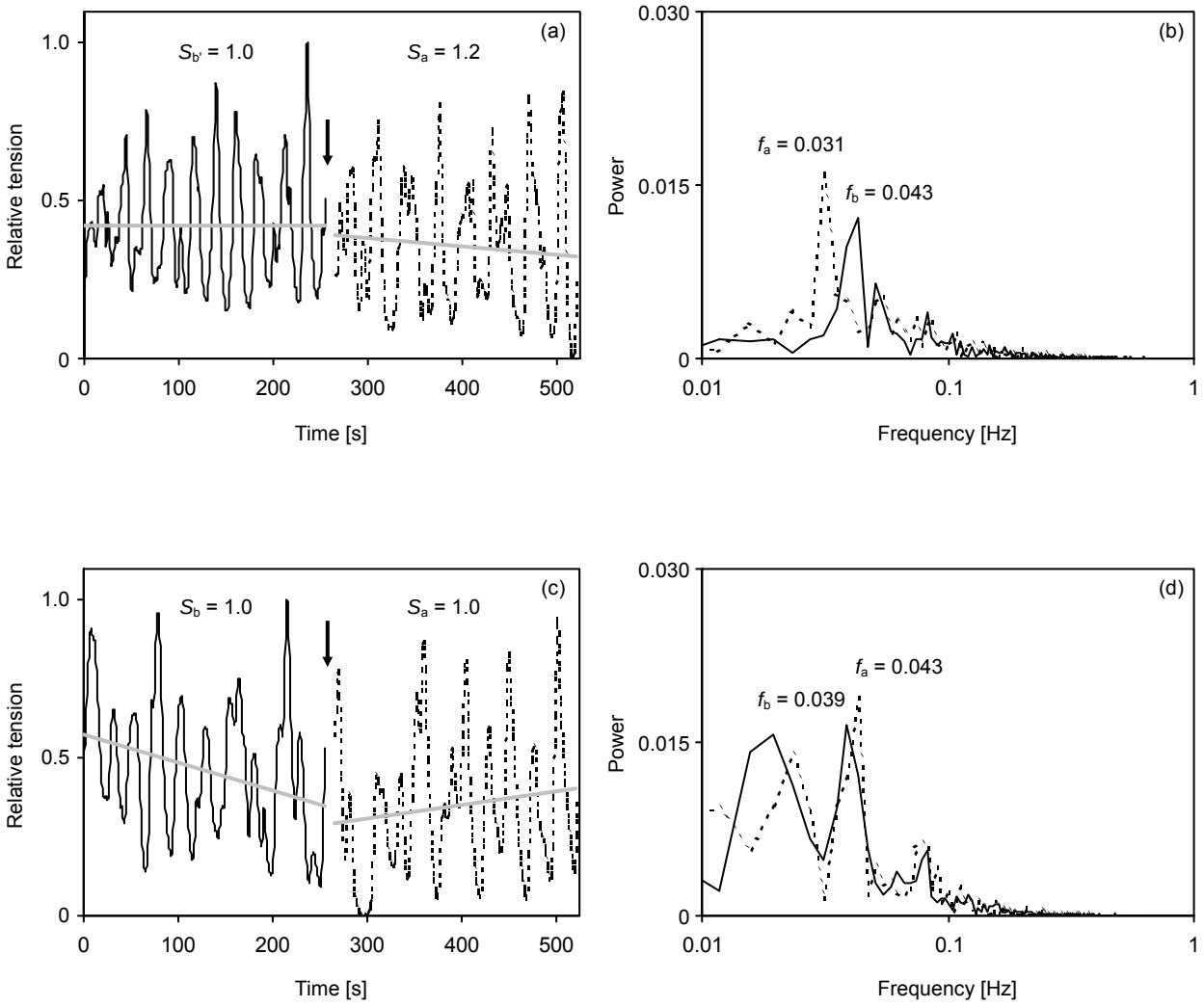

Fig. 8.3 Effects of (a) bifenazate and (c) diazene at 10^{-6} M on the contraction of the *Leucophaea maderae* hindgut previously bicuculline methiodide (BMI) treated. The concentrations of BMI in the bifenazate and diazene treatments were 10^{-9} M and 10^{-8} M, respectively. Power spectra (b) and (d) were calculated by the fast Fourier transform (FFT) of wave signals (a) and (c), respectively. Solid and broken lines indicate before and after applying chemicals, respectively. Arrows indicate the time of application of a chemical. The S_b and S_a indicate an index of the amplitude of the hindgut contraction before and after applying chemicals, respectively. The f_b and f_a indicate the peak frequency of the hindgut contraction before and after applying chemicals, respectively. The gray lines were calculated by a regression analysis.

Discussion

The mode of actions of bifenazate and diazene were investigated by the hindgut contraction assay. It is suggested that bifenazate apparently acts on the GABAR in the nervous system (Dekeyser, 2005). GABARs are abundant in both vertebrates and invertebrate where they mediate predominantly inhibitory neurotransmission (Millar et al., 1994). Insect GABARs are the action site of several important insecticides including cyclodiene, i.e., polychlorocycloalkanes (dieldrin and lindane) and phenyl pyrazoles such as fipronil (Rauh et al., 1990; Colliot et al., 1992; Anthony et al., 1993; ffrench-Constant et al., 2000).

Vertibrate GABARs have been classfied into three pharmacologically distinct subtypes, the $GABA_AR$, $GABA_BR$, and $GABA_CR$ (Johnston, 1986). The vertebrate $GABA_AR$ and $GABA_CR$ ubtypes are integral GABA-gated ion channels that are selectively permeable to chloride and bicarbonate ions, whereas $GABA_BRs$ modulate either calcium or potassium channels coupled with a G-protein-linked second messenger system (Bowery et al., 2002; Bettler et al., 2004).

When the heart is exposed to GABA, the heart beat amplitude and frequency decreased in the horseshoe crab *Limulus polyphemus* (Pax and Sanborn, 1967; Abbott et al., 1969; Benson, 1989). In the circular smooth muscles of the rat distal colon, GABA inducted a transient monophasic relaxation at low concentrations or a biphasic effect characterized by a relaxation followed by a tonic contraction at high concentrations (Bayer et al., 2002). These authors suggested that $GABA_ARs$ were involved in the regulation of spontaneous mechanical activities in the circular smooth muscles.

A *Drosophila* GABA-gated chloride channel subunit cDNA has been cloned by genetic mapping of a mutation underlying insecticide resistance in the *Drosophila*

mutant *Rdl* (*Resistant to dieldrin*) (ffrench-Constant et al., 1991). In all of the cloned GABAR homologues from insects and ticks, resistance to cyclodiene insecticide is based on a serine (S) or glycine (G) residue substitution at the second transmembrane domain, known as the *Rdl* mutation site responsible for binding pesticides (ffrench-Constant et al., 1993, 1994, 2004; Thompson et al., 1993; Kaku and Matsumuma, 1994; Miyazaki et al., 1995; Wolff and Wingate, 1998; Zheng et al., 2003; Bass et al., 2004).

Recently, a novel GABAR has been cloned from *T. urticae*; the deduced amino acid sequence displays 68% similarity to the *Drosophila* RDL and possesses a histidine (H) instead of the more common S or G (Canlas, 2005). This supports the GABA receptor hypothesis for the mode of action of bifenazate and can explan its specific toxicity against *T. urticae*.

Although bifenazate has low toxicity to the cockroach *L. maderae*, the present data show that bifenazate and diazene possessed the binding site to induce inhibitory effects on intensity and rhythm of the hindgut contraction of *L. maderae* (Fig. 8.1). Although diazene toxicity to *T. urticae* is higher than bifenazate (Canlas, 2005; Ochiai et al., 2007), the inhibitory effects of bifenazate on the hindgut contraction were higher than those of diazene from a comparison of EC_{50} values (Fig. 8.2). It is known that diazene is an unstable compaund. Therefore, the difference between effects of diazene on the hindgut contraction and its toxicity might be caused by its unstability.

The inhibitory effects of bifenazate and diazene on the hindgut contraction were blocked by the GABAR antagonist BMI (Fig. 8.3). This indicates that the inhobition of the hindgut contraction may be controlled by GABARs at the visceral neuromuscular junction.

The present study supports the GABAR hypothesis in the bifenazate mode of action and suggests that the target-site of diazene is also in GABARs. However, effects of chemicals, which act on the respiratory pathway in the mitochondria of spider mites, as described by Van Leeuwen et al. (2006, 2007, 2008), should also be investigated to approach the true mode of action of bifenazate.

Chapter 9

General Discussion

1 Photoperiodism

Organisms regulate their behavioral and developmental programs to maximize resource utilization and minimize hazard risks. If resource availability and hazard occurrence fluctuate in a cyclical manner, the best adaptation for survival is synchronization of their internal rhythms to the environmental periodicity.

Ever since life first appeared on this planet, it has been periodically exposed to solar radiation containing ultraviolet (UV) radiation. Probably, at the earliest times when the protective ozone layer was not formed around the earth, organisms were required to avoid intense UV-C as well as UV-B that directly damage DNA and produce harmful reactive oxygen species (ROS). Therefore, light or dark must be recognized by organisms living at the place where solar radiation reaches. Probably, this pressure induced evolution of photoreceptors and photoreceptors developed by increasing visual sensation. In “Light Switch” theory (Parker, 2003), active predation became possible with the advent of vision, and prey species found themselves under extreme pressure to adapt in ways that would make them less likely to be spotted. According to this theory, the Cambrian Explosion was induced by acquisition of vision.

On this background, the circadian oscillation to modulate light signal from photoreceptors must have been evolved (possibly before acquisition of vision). Photoperiodism is an adaptation to track passage of seasons by measuring photoperiod that provides an accurate seasonal signal (Tauber et al., 1986).

Namely, organisms have been adapted to daily and seasonal rhythms of the environment by developing circadian oscillation and photoperiodism, respectively, in locomotion, feeding, development, reproduction, and morphogenesis (Saunders, 2003). Mathematical models using a number of insect systems have been developed that attempt to causally relate the circadian oscillation to photoperiodic time measurement (Takeda and Skopik, 1997). Although molecular mechanisms of the circadian time measurement system (circadian clock) have been gradually revealed in the prokaryotic cyanobacterium *Synechococcus elongatus* as well as the eukaryotic systems *Neurospora crassa*, *Drosophila melanogaster*, *Arabidopsis thaliana*, and mammals by using a variety of molecular probes (e.g. Mackey, 2007; Kuhlman 2007), molecular investigations on photoperiodism are still outdistanced. Photoperiodism is not only an attractive scientific theme but also an important key for management of pests since it is a critical determinant of the life history.

The two-spotted spider mite, *Tetranychus urticae* is one of the most serious phytophagous pests and the developmental switch between diapause and reproduction is photoperiodically regulated. Veerman and his colleagues made a formal analysis on the photoperiodic clock in *T. urticae*. They constructed a photoperiodic model based on three characteristics (1) a non-repetitive long-night measuring photoperiodic clock, which may be either an hourglass or a rapidly damped oscillator; (2) since Nanda-Hamner protocol produced rhythmic fluctuations in diapause induction, circadian system is somehow involved in the photoperiodic response, and (3) the so-called photoperiodic counter that accumulates information from the photoperiodic clock (Vaz Nunes and Veerman, 1982, 1986; Veerman and Vaz Nunes, 1987; Veerman, 2001; Veerman and Veenendaal, 2003). However, the basic knowledge on several physiological aspects, particularly, hormonal mechanism of diapause regulation,

molecular mechanism of circadian system and its involvement in photoperiodism, and photoreception mechanism still remain unclarified.

By using a technology of light-emitting diodes (LEDs), a space-saving device "LED bottle" were developed for experimental investigations on photoperiodism in this mite (Fig. 2.1) (Suzuki et al., 2007), and the results revealed that blue light was most effective in photoperiodic determination in *T. urticae* (Fig. 3.2) (Suzuki et al., 2008a). This indicates the existence of a blue light receptor for the photoperiodic system, suggesting a utility of blue light in manipulating photoperiodism of *T. urticae* as a physical control agent.

An action spectrum for suppressing the activity of the melatonin synthetic enzyme, arylalkylamine *N*-acetyltransferase (NAT), which has been believed to function in the circadian system in mammals, showed spectral sensitivity with the main maximum at 350 nm and the second maximum at 450 nm (Fig. 5.4) (Suzuki et al., 2009). This is similar to the absorption spectra of *Drosophila* cryptochrome (dCRY) that is photoreceptor transducing a blue light signal to the circadian clock system in *D. melanogaster* (Berndt et al., 2007) and to the spectral sensitivity for photoperiodism in *T. urticae* (Suzuki et al., 2008a). Therefore, cryptochrome may be the circadian photoreceptor and be involved in the photoperiodic time measurement in *T. urticae*.

Lees (1981) conducted a light-break protocol with different monochromatic lights applied at different phase of dark period and suggested that at least two photoreceptors (short- and long-wavelength types) function in photoperiodism in the aphid, *Megoura viciae*, which has a similar photoperiodic system to *T. urticae*. Therefore, the light-break protocol should be conducted to confirm another type (long-wavelength type) of photoreceptor for photoperiodism of *T. urticae*.

In *Drosophila*, circadian genes such as *per*, *timeless*, *Clock*, *cycle*, *cryptochrome*, *vrille*, *Pdp1*, *doubletime*, *shaggy*, *casein kinases I* and *II*, and *cwo* (*clockwork orange*) have been found and a sophisticated negative feedback model has been constructed (e.g. Kadener et al., 2007; Lim et al., 2007; Sandrelli et al, 2008). However, molecular approaches to the circadian system and photoperiodism in mites have not been conducted, and therefore the mechanism remains as a black box. Further experiments, both as phenomenological and molecular approaches are required to clarify a mechanism of the mite photoperiodism.

In conclusion, the data suggested that short-wavelength light can be useful for photoperiodic control (disturbance of photoperiodism) in *T. urticae*. When photoperiodic control is applied in the management of *T. urticae*, photoperiodism in phytoseiid mites (for overview, see Veerman, 1992) as well as plant growth must be investigated for the harmonious implementation of IPM with biological control.

2 UV sensitivity

Organisms have evolved physiological mechanism to protect themselves from UV radiation such as circadian system, pigmentation, and repairing system of damaged DNA/RNA, especially when they colonized the land habitat.

Melatonin can serve one possible system for protection against UV damage on membrane, particularly lipids. UV radiation, particularly UV-B, is absorbed by some coenzymes and pigments in vivo, raising these molecules to an excited-state; the excitation energy is finally transferred to H_2O molecules yielding ROS. It has been suggested that the UV-B-induced formation of ROS can induce damage to the DNA (Beehler et al., 1992) and activate the transcriptional factor activating protein (AP)-1

(Devary et al., 1992). In mammals, AP-1 seems to promote the transcription of arylalkylamine *N*-acetyltransferase (NAT) (Estrada-Rodgers et al., 1998) and 5-hydroxyindole-*O*-methyltransferase (HIOMT) (Rodriguez et al., 1994), which are key enzymes for melatonin synthesis. Melatonin functions as a ROS scavenger (Reiter et al., 2000). Therefore, AP-1 may transmit UV signals to the system for melatonin synthesis to remove ROS. Actually, the melatonin concentration in the roots of the plant *Glycyrrhiza uralensis* is increased by UV-B irradiation (Afreen et al., 2006). The ROS removal system by melatonin might be an evolutionarily conserved utility shared both by animals and plants.

Under UV-B (300 nm), the NAT activity and melatonin content were suppressed at low doses but elevated at high doses in survived adult females of *T. urticae* (Figs. 7.2, 7.3) (Suzuki et al., 2008b). Elevation of the NAT activity and melatonin content by high dose UV-B irradiation may indicate that the UV-B signals induce melatonin synthesis for ROS removal also in mites.

In non-diapause females of *T. urticae*, the mortality and escape were induced and the oviposition was suppressed under UV-B at high doses (Figs. 6.3, 6.4) (Suzuki et al., in preparation). However, no inhibitory effect on mortality was observed in diapause females but most of them attempted to escape from UV-B (Tables 6.3, 6.5). Most likely, non-diapause females on the underside of leaves avoid UV-B by using leaves as an UV-B cut filter in summer. Further behavioral assay should be conducted to prove this UV-B hypothesis explaining the reason why non-diapause females inhabit on the underside of leaves. On the other hand, high escape rate and low mortality observed in diapausing females suggest that diapausing females have at least two lifelines: (1) emigration to UV-free environment and (2) carotenoid accumulation, to overcome deleterious effects due to UV radiation for several months in winter.

In conclusion, UV-B can be used as an effective tool in physical control of *T. urticae* and the mite might develop phenological adaptation to UV-B environment by regulating behavior, entering diapause, forming carotenoids, and the upregulation of melatonin synthesis that provides protection against UV/ROS damage.

3 GABA receptor

A novel carbazate acaricide, bifenazate, possesses specific toxicity to *T. urticae* (Dekeyser et al., 1996). Since bifenazate shows no cross-resistance to spider mites resistant to a wide range of conventional acaricides (Grosscurt and Avella, 2005; Pree at al., 2005; Van Leeuwen et al., 2005; Ochiai et al., 2007), its mode of action may distinct from that of other acaricides. Although no detailed investigation on the mode of action has been reported, preliminary studies suggest that bifenazate apparently acts on the post-synaptic γ-aminobutyric acid (GABA) receptor (GABAR) in the nervous system (Dekeyser, 2005).

I pharmacologically approached to the bifenazate mode of action by the cockroach hindgut assay. In the result, bifenazate and principal active metabolite, diazene, possessed inhibitory effects on contraction intensity and rhythm in the hindgut (Fig. 8.1). Moreover, the inhibitory effects of bifenazate and diazene were blocked by the GABAR antagonist, bicuculline methiodide (Fig. 8.3). This suggests that bifenazate and diazene stimulate the GABAR function and supports the GABAR hypothesis in the bifenazate mode of action.

Ion channels and neurotransmitter receptors such as voltage-gated sodium channels, GABARs, and nicotinic acetylcholine (ACh) receptors (nAChRs) are the major target sites of insecticides (Narahashi et al., 1999). Moreover, the world's three

bestselling pesticides target three specific receptors, namely, inhibitory glutamate receptors (GluRs), nAChRs and GABARs (Eguchi et al., 2007).

Inhibitory GluRs, nAChRs, and GABARs are members of the Cys-loop superfamily (Sine and Engel, 2006) of ligand-gated ion channels, which includes both cation-permeable and anion-permeable channels (Raymond and Sattelle, 2002); therefore, inhibitory GluRs and GABARs are referred to as glutamate-gated chloride channels and GABA-gated chloride channels, respectively.

Vertibrate GABARs have been classfied into three pharmacologically distinct subtypes, the $GABA_AR$, $GABA_BR$, and $GABA_CR$ (Johnston, 1986). The vertebrate $GABA_ARs$ and $GABA_CRs$ subtypes are above-mentioned GABA-gated chloride channels, whereas $GABA_BRs$ modulate either calcium or potassium channels through a G-protein-linked second messenger system (Bowery et al., 2002; Bettler et al. 2004).

Insects also have both ionotropic and metabotropic GABARs (Buckingham and Sattelle, 2004). Insect GABARs are found not only in the central nervous system (CNS) but also in the neuromuscular junction (Lummis, 1990). Insect ionotropic GABARs functionally resemble vertebrate $GABA_ARs$, although they have a unique pharmacology (Buckingham et al., 2005). A gene encoding a GABAR subunit was first isolated from *Drosophila melanogaster* and named *Rdl* (*Resistant to dieldrin*). The so-called *Rdl* mutation confers resistance to the cyclodiene insecticide dieldrin (ffrench-Constant et al., 1991).

The *Rdl*-coding protein (RDL) is probably a common subunit of insect ionotropic GABARs as the orthologs have been identified from other arthropod species such as Diptera (*Aedes aegypti*, *D. simulans*, *Lucilia cuprina*, *Musca domestica*, *Ceratitis capitata*), Lepidoptera (*Heliothis virescens*, *Spodoptera exigua*, *Plutella xylostella*, *Bombyx mori*), Coleoptera (*Tribolium castaneum*), Hemiptera (*Laodelphax striatella*),

Hymenoptera (*Apis mellifera*), Blattodea (*Blattella germanica*), Copepoda (*Lepeophtheirus salmonis*), and Acarina (*Dermacentor variabilis*) (GenBank, NCBI). RDL-containing GABARs were shown to be involved in mediating synaptic inhibition in *D. melanogaster* neuronal circuits (Lee et al., 2003).

Two other GABAR-like subunits, GRD (Harvey et al., 1994) and LCCH3 (Henderson et al., 1993, 1994; Hosie et al., 1997), were cloned from *D. melanogaster*. RDL not only forms a homopentamer that imitates the pharmacology of native GABARs, but also forms a heteromer with LCCH3 (Zhang et al., 1995). In addition to this variation, alternative splicing and RNA editing of *Rdl* further increase the complexity of insect GABAR functions and pharmacology (ffrench-Constant and Rocheleau, 1993; Hosie et al., 2001; Buckingham et al., 2005; Es-Salah et al., 2008). Metabotropic $GABA_BRs$ were also identified in *D. melanogaster* and partially characterized (Bai and Sattelle, 1995; Mezler et al., 2001).

A novel GABAR (TuGABAR) was cloned and characterized from *T. urticae* by Canlas (2005). The clone was 2266 nucleotides long including the 87 bp 5' untranslated region (UTR), 421 bp 3' UTR, and 1755 bp open reading frame (ORF) that encoded for 585 amino acid residues. The TuGABAR amino acid sequence shared 68% similarity with the *Drosophila* RDL. Typical GABAR characteristics: Cys-loop disulphide bridge, putative extracellular region, and four transmembrane domains (M1–4) were conserved in the TuGABAR. In all the cloned GABAR homologues from arthropods, resistance to cyclodiene insecticides was based on a serine (S) or glycine (G) residue at the M2 domain, which is known as the *Rdl* mutation site responsible for binding pesticides. Interestingly, the spider mites (resistant to cyclodienes) possess a histidine (H) instead of the more common S or G residue. Histidine being a charged amino acid may introduce a change in the net charge that

could alter polarity and mediate resistance to cyclodienes and specific toxicity of bifenazate to *T. urticae*.

In conclusion, the GABAR hypothesis on the bifenazate mode of action was corroborated by the above-mentioned pharmacological approach. Further investigations by functional analysis of the TuGABAR will open a new horizon for study on the bifenazate mode of action and construct a novel screening system for TuGABAR targeted acaricides.

4 Concluding remarks

Taken together all the data obtained in this study can contribute the implementation of integrated management of *T. urticae* for the reason of following possibilities: (1) disturbance of the photoperiodic induction of diapause by blue light irradiance, (2) inhibition of the population growth by UV-B irradiation, (3) combined usage of predators and *T. urticae*-specific acaricides such as bifenazate, and (4) development of effective acaricides targeting the melatonin system (mediating the circadian and ROS removal systems) and GABAR in *T. urticae*.

Suzuki, T. (2009) Integrated management of the two-spotted spider mite, *Tetranychus urticae*: photoperiodism, UV sensitivity and GABA receptor. Doctoral Dissertation, Kobe University, 146 pp.

Summary

1. The two-spotted spider mite, *Tetranychus urticae* is distributed worldwide and is one of the most serious pests for fruit trees, vegetables, and ornamental plants. Conventionally, chemical control using acaricides has played the central role in management of *T. urticae*. However, frequent use of acaricides often induces resistance development supported by a high rate of population growth in *T. urticae*. This leads to a breakdown in population management of *T. urticae*. Therefore, the implementation of integrated pest management (IPM)—an approach that employs a combination of existing pest management measures including timely application of small amounts of chemicals—is urgently needed. For the implementation of IPM in spider mite control, I investigated photoperiodism (Chapters 2–5), sensitivity to ultraviolet (UV) radiation (Chapters 6 and 7), and γ-aminobutyric acid (GABA) receptor (Chapters 8) in *T. urticae*. General introduction and discussion of these studies were described in the Chapters 1 and 9.

2. Photoperiodism as well as growth rate are important determinants in life history. Diapause in *T. urticae* is induced at the adult stage by short-day conditions to overwinter. Therefore, disturbance of photoperiodic induction of diapause in *T. urticae* by manipulating light environments would lead to non-chemical control of the mite. Utilizing a technology of light-emitting diodes (LEDs), I developed a LED bottle, which provides efficient a photoperiodic experiment, and revealed that diapause in *T. urticae* was induced by providing light period of 24 h or shorter with 16 h dark period (Chapter 2). These photoperiods might support plant growth but discourage an increase in the mite population.

3. Spectral sensitivity for photoperiodic induction of diapause in *T. urticae* was also investigated by utilizing the LED technology (Chapter 3). Within the wavelength range of visible light, blue light was most effective and red light has no effect on photoperiodic induction of diapause. This must contributes to the decision on a light source, which is most effective in artificial control of the mite photoperiodism.

4. The deutonymphal stage was most sensitive to photoperiods for determination of reproduction or diapause (Chapter 4). Moreover, nymphal development, particularly, in the deutonymphal stage was inhibited by diapause-inducing photoperiods. This suggests that a drastic physiological change for photoperiodic determination of reproduction or diapause occurred during deutonymphal stage and that manipulation of photoperiod can also be used in the reduction of the rate of nymphal development, which is a most important parameter in the mite population growth.

5. An action spectrum for the activity suppression of the melatonin synthetic enzyme, arylalkylamine *N*-acetyltransferase (NAT), which has been believed to function in the circadian system in mammals, was examined by using the Okazaki Large Spectrograph (OLS) (Chapter 5). The action spectrum showed spectral sensitivity with the main maximum at 350 nm and the second maximum at 450 nm. This is similar to the absorption spectrum of the blue-light receptor, cryptochrome of *Drosophila* and above-mentioned spectral sensitivity for photoperiodic induction of diapause in *T. urticae*. Therefore, cryptochrome may be the circadian photoreceptor and be involved in the photoperiodic time measurement in *T. urticae*.

6. UV radiation would induce lethal oxidative damages particularly in small organisms such as insects and mites. Therefore, UV irradiation is considered as an attractive alternative measure for non-chemical control of *T. urticae*.

6-1. Sensitivity and phenological adaptation to UV radiation in *T. urticae* was examined by exposing them to UV-C (250 nm), UV-B (300 nm) and UV-A (350 nm) using the OLS (Chapter 6). In non-diapause females, both UV-C and UV-B induced a high mortality and escape, and inhibited oviposition, while such effects were not observed under UV-A. The deleterious effects of UV-C were higher than those of UV-B, while no UV-C reaches to the earth surface. Under UV-B, the ED_{50} values, where 50% of mortality plus escape rate and 50% of oviposition rate in control (continuous darkness) were induced, were 104 and 41 kJ m^{-2}, respectively. The ED_{50} values are comparable to the doses observed in the field for 2–5 days in summer when the mites inhabit the underside of leaves with dense vegetation. These suggest that the mites use leaves as an UV-B cut filter to avoid the deleterious effects and that UV-B is an effective tool for the mite control.

6-2. In diapausing females, low mortalities were observed under all types of UV radiation even at high doses. Interestingly, more than half of diapausing females escaped under all types of UV radiation even at low doses. The orange body color in diapause females is known as a result of accumulation of carotenoids that plays the role as a scavenger of UV-induced reactive oxygen species (ROS). There suggest that diapausing females have at least two lifelines: (1) emigration to no UV environment and (2) carotenoid accumulation, to overcome deleterious effects of UV radiation for several months in winter.

7. Melatonin serves as a scavenger of ROS, which is produced by UV irradiation, in mammals. Changes in NAT activity and melatonin content were determined in *T. urticae* after irradiating them with UV-B (300 nm) and UV-A (350 nm), blue (450 nm) and red light (650 nm) using the OLS (Chapter 7). Elevations of the NAT activity and melatonin content were observed under high intensity UV-B and UV-A irradiation. This supports the notion that the UV signals initiate melatonin synthesis for ROS removal in mites and that the ROS removal system is an evolutionarily conserved among organisms.

8. A hypothesis that mode of action of the species-specific acaricide, bifenazate, is related to the post-synaptic γ-aminobutyric acid receptor (GABAR) was supported by the hindgut contraction assay the cockroach, *Leucophaea maderae* (Chapter 8). The intensity and rhythm of the hindgut contraction were inhibited by applying bifenazate and its principal active metabolite, diazene. The inhibitory effects were blocked by the GABAR antagonist, bicuculline methiodide. These data suggest that bifenazate and diazene stimulate the GABAR function, supporting the GABAR hypothesis in the bifenazate mode of action.

9. Taken together, I propose several measures for implementation of the integrated management of *T. urticae* as follows: (1) disturbance of the photoperiodic induction of diapause (overwintering) of adult females by exposing them to blue light, (2) inhibition of the population growth by exposing mites, which inhabit on the underside of leaves, to solar radiation including UV-B using reflection materials, (3) the combination usage of predatory mites and *T. urticae*-specific acaricides such as bifenazate, and (4) development of effective acaricides targeting the melatonin system (mediating the circadian and ROS removal systems) and GABAR in *T. urticae*.

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List of Publications

Suzuki, T., H. Amano, E. Goto, M. Takeda and T. Kozai (2007) Effects of extending the light phase on diapause induction in a Japanese population of the two-spotted spider mite, *Tetranychus urticae*. *Experimental and Applied Acarology* 42, 131–138, DOI: 10.1007/s10493-007-9083-0.

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