Eur. J. Entomol. **107**: 687–690, 2010
http://www.eje.cz/scripts/viewabstract.php?abstract=1579
ISSN 1210-5759 (print), 1802-8829 (online)

An improved method for the culture of wing discs of the wingless bagworm moth, *Eumeta variegata* (Lepidoptera: Psychidae)

SHUHEI NIITSU[1] and SAORI LOBBIA[2]

[1]Department of Life Science, International Christian University, Osawa 3-10-2, Mitaka, Tokyo 181-8585, Japan; e-mail: shu-30@aioros.ocn.ne.jp

[2]Department of Integrated Biosciences, Graduate School of Frontier Sciences, University of Tokyo, Bioscience Building 501, Kashiwanoha, Kashiwa, Chiba 277-8562, Japan

Key words. Lepidoptera, Psychidae, *Eumeta variegata*, bagworm moth, wing disc, tissue culture, differentiation

Abstract. Adults of the wingless bagworm moth, *Eumeta variegata*, show remarkable sexual dimorphism. Final-instar larvae of the male have invaginated wing discs, whereas those of the female are rudimentary. To determine the best method for culturing the wing discs of *E. variegata*, which in both sexes are attached to the larval integument, two methods of culturing the larval wing discs are compared. Initially, a stationary culture was used. In these cultures necrotic cells and degeneration of wing discs of males were sporadically observed. By contrast, many small vacuoles were observed in the female wing rudiment under these conditions. In order to overcome some of the problems associated with stationary culture, rotating culture was used and resulted in the wing discs of males and females remaining in good condition. A histological analysis revealed that the wing disc morphology was normal when they were cultured in this way. These results indicate that rotating the culture medium is the better procedure for studying the action of hormones on the differentiation and metamorphosis of reduced wing rudiments in *E. variegata*.

INTRODUCTION

Tissue culture in vitro is an effective method for investigating the responses to hormone and physiology of internal organs. Culture of wing discs in vitro has been used to study the role of ecdysteroid in differentiation and its mode of action on insect tissues (Kawasaki, 1989). Several authors have studied the differentiation of wing discs of Lepidoptera in vitro (Oberlander et al., 1973; Nardi & Willis, 1979; Blais & Lafont, 1980; Fujiwara & Ogai, 2001); however, there are few endocrinological studies on wing discs cultured in vitro of wingless or brachypterous holometabolous insects. Indeed, in only two studies on the wingless females of the tussock moths (Lobbia et al., 2003, 2007) were the endocrinological mechanisms investigated. Studying the endocrinological mechanisms of wing reduction might help in understanding the regressive evolution of wings in holometabolous orders.

Eumeta variegata is one of the bagworm moths of the family Psychidae, the adult females of which lack wings and live throughout their life in the larval case, whereas adult males have functional wings. Final-instar larvae of the male have normal wing discs, whereas those of the female are rudimentary (Niitsu, 2003). For Lepidoptera, there are several reports on the developmental processes of wingless or brachypterous moths (Fedotov, 1939; Nardi et al., 1991; Niitsu, 2001, 2003; Niitsu & Kobayashi, 2008). During a preliminary study, it proved difficult to remove the minute wing rudiments from the body of a female, so it was decided to develop a method for culturing pieces of larval integument. The system used to culture larval integument for experiments on the role of ecdysone and juvenile hormone in larval-larval apolysis and larval-pupal transformation in *Manduca sexta* by Riddiford (1976) was adopted. It was hypothesized that the development and reduction of wings is similarly controlled by two hormones, i.e., ecdysteroid and juvenile hormone. However, the cellular and physiological mechanisms involved in wing reduction are poorly understood. Before initiating the tissue culture experiments, the optimal culture conditions for the histological analysis of wing discs in vitro were established.

In this study, the differential development of the wing discs of *E. variegata* in stationary and rotating cultures were compared. The rotating culture provided the best conditions for normal development, which was confirmed by the histological studies.

MATERIAL AND METHODS

Insect samples

Larvae of *E. variegata* were collected from roadside trees in Tokyo, Japan. All these larvae reached the last-larval instar and then spent the winter in diapause. At this stage, female larvae are typically larger than males, so it was possible to distinguish the sexes simply by the size of the larvae.

Dissecting and culturing tissue

Larvae were surface-sterilized in 70% ethanol for 5 min and dissected across the mesothorax to locate the forewing discs. The wing discs, with adjacent larval integument, were excised from the body using a scalpel and rinsed three times in cold phosphate-buffered saline (PBS; 137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, 1.47 mM KH_2PO_4, pH 7.4). In static culture, isolated wing discs were cultured at 25°C on the surface of 1.0 ml of Grace's medium (Gibco BRL, Grand Island, NY, USA), containing an antibiotic-antimycotics (Gibco BRL), in a 4-well multi dish (Nalge Nunc, Rochester, NY, USA). In rotating culture, wing discs plus larval integument were cultured on the surface of 1.0 ml Grace's medium (Gibco BRL) containing an antibiotic-antimycotics (Gibco BRL) in 1.5-ml centrifuge tubes. The tissue was incubated and gently rotated (15 rpm) for 72 h at 24–25°C. Rotation was incorporated into the protocol so that the larval integument was frequently exposed to air during incubation.

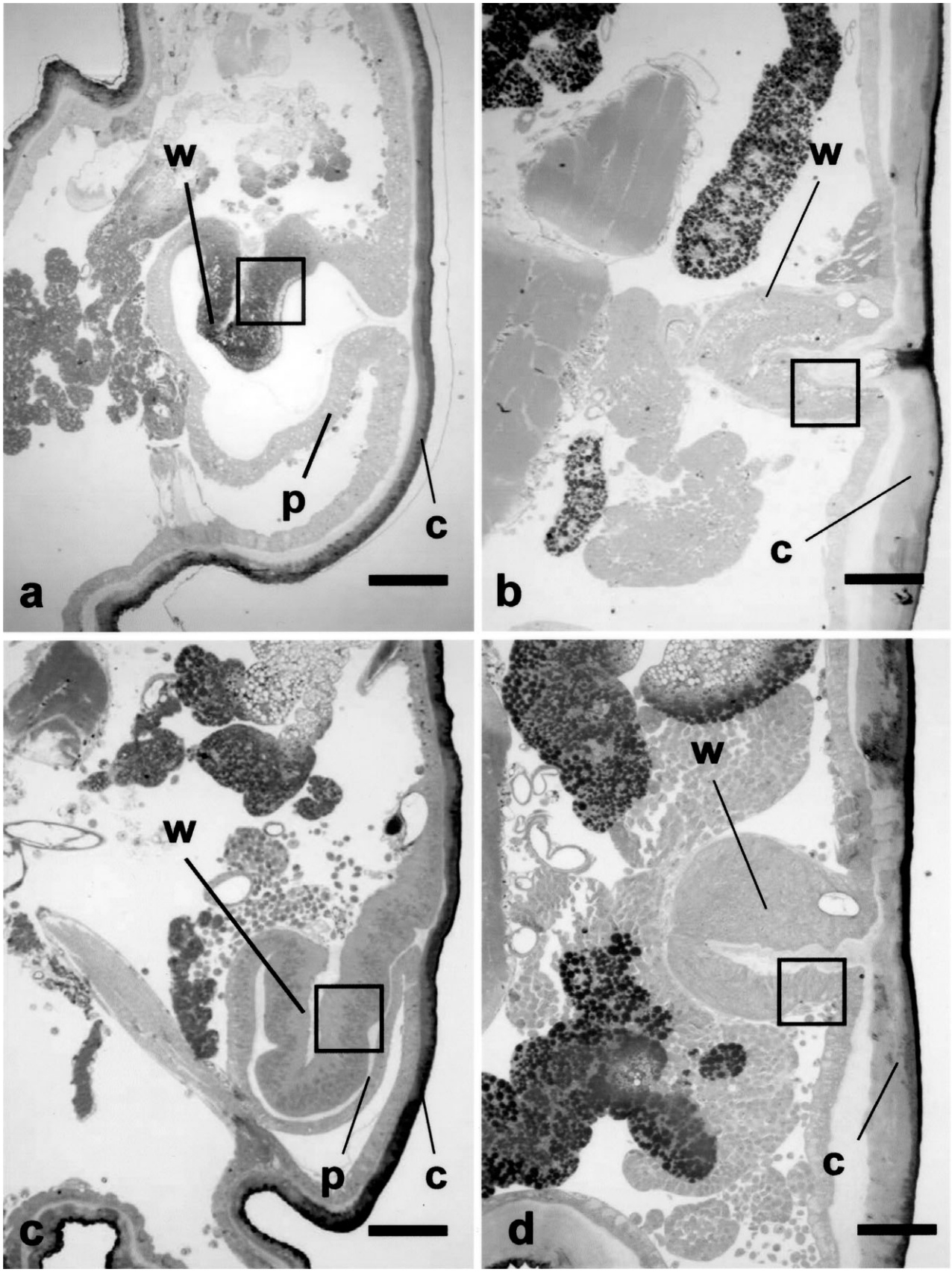

Fig. 1. Cross-sections of male and female wing discs of *Eumeta variegata* cultured without hormone treatment for 72 h. a – male wing disc kept in static culture. The boxed area in a corresponds to Fig. 2a. b – female wing rudiment kept in static culture. The boxed area in b corresponds to Fig. 2b. c – male wing disc kept in rotating culture. The boxed area in c corresponds to Fig. 2c. d – female wing disc kept in rotating culture. The boxed area in d corresponds to Fig. 2d. Lettering: w – wing disc (male) or wing rudiment (female); c – larval cuticle; p – peripodial epithelium. Scale bars: 100 μm.

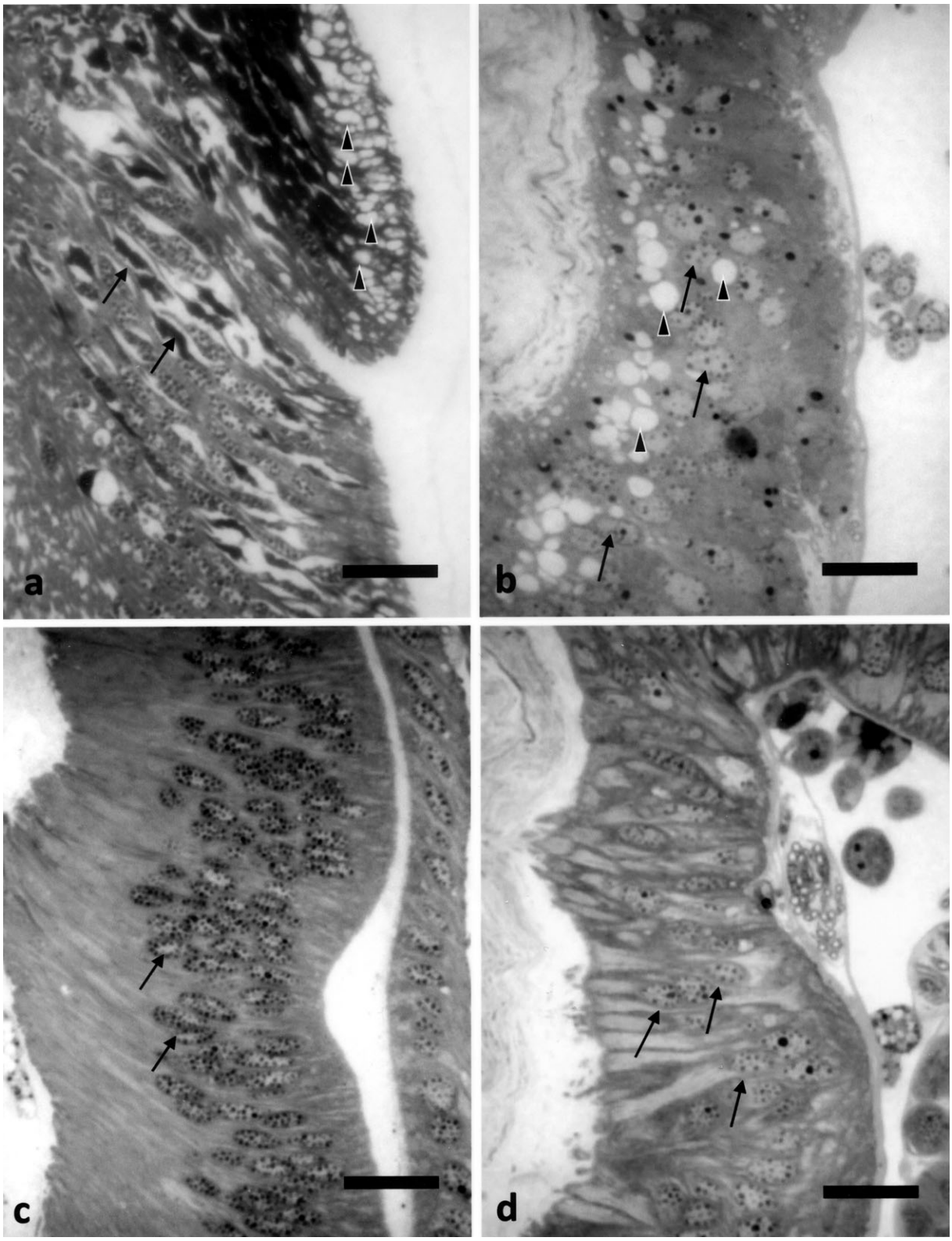

Fig. 2. High magnification photographs of the cross-sections shown in Fig. 1 (a, b – discs maintained in stationary culture; c, d – in rotating culture). a – male wing disc cultured in a medium without hormone. Note the scattered necrotic nuclei (arrows) and small vacuoles (arrowheads). b – cultured female wing rudiment. Note the many small vacuoles (arrowheads). Arrows indicate nuclei. c – cultured male wing disc. Nuclei in the cells of the wing epithelium are normal (arrows). d – female wing rudiment. After culture, the rudiment consists of a single layer of cells with normal nuclei (arrows). Scale bars: 20 μm.

Preparation of tissue

The tissues cultured in vitro were fixed first in Karnovsky's fixative (2% paraformaldehyde + 2.5% glutaraldehyde) and then in 2% osmium tetroxide. After dehydration through a series of ethanol and propylene oxide, the wing discs were embedded in Epon 812 (TAAB Laboratories Equipment Ltd., Aldermaston,

UK). Semithin sections (1 μm thick) were cut using a rotary microtome, mounted on microscope slides and then stained with Azur-B. These sections were observed under a light microscope.

RESULTS AND DISCUSSION

To establish a tissue culture method for studying wing disc development in vitro, the wing discs of both sexes were initially placed in Grace's medium without hormone for 72 h. The wing discs of males atrophied under these conditions and small vacuoles were sporadically observed in the epithelial cells of the wings (Fig. 1a), which underwent necrosis (Fig. 2a, arrows). In contrast, under these conditions, many vacuoles but no necrosis was detected in the epithelial cells of female wing rudiments (Fig. 1b, 2b).

When maintained in rotating culture, without hormone treatment for 72 h, there were no small vacuoles in cells of the male and female wing discs (Fig. 1c, d). There was no cell proliferation in the female wing discs (Fig. 2d) and the internal structures of the epithelium of the male wing retained a smooth cell arrangement (Fig. 2c). These results are consistent with those of a previous study on the development of wing discs in *E. variegata* under natural conditions (Niitsu, 2003). This indicates that wing discs in rotating culture develop more normally than those maintained in stationary culture (Fig. 1d). As indicated in Fig. 1c and 1d, the larval integuments attached to the wing discs remained in good condition for up to 72 h. This in vitro culture system has been used to show that 20-hydroxyecdysone (20E) induces the sex-specific differentiation of wing discs in *E. variegata* (Niitsu et al., 2008).

Thus, there is a simple and effective method for culturing the wing rudiments and attached larval integument of *E. variegata*. In rotating culture conditions there is apparently a greater nutrient availability and more efficient transfer of oxygen than in stationary culture. It is reported that a high partial pressure of oxygen is needed for wing development (Kawasaki, 1989). However, wing development in *E. variegata* occurs in rotating cultures on media containing 1 μg/ml 20E and exposed to ambient levels of oxygen (Niitsu et al., 2008).

In conclusion, the histological analysis showed that the morphology of the wing discs of *Eumeta variegata* cultured for 72 h using the rotating tissue culture method was normal. This method provides a good method of culturing both the male wing discs and female wing rudiments, and associated larval integument, of *E. variegata*. Further studies are needed to clarify the precise response to hormones during the larval-pupal transformation in wingless female bagworm moths.

ACKNOWLEDGEMENTS. We thank H. Fujiwara and R. Futahashi (University of Tokyo) for his help with the experiments and Y. Kato and T. Kamito (International Christian University, Tokyo, Japan) for their helpful comments during the preparation of the manuscript.

REFERENCES

Blais C. & Lafont R. 1980: In vitro differentiation of Pieris brassicae imaginal wing discs: Effects and metabolism of ecdysone and ecdysterone. *Wilhelm Roux's Arch*. **188**: 27–36.

Fedotov D.M. 1939: On the phenomenon of regressive changes in some bagworm moth (Psychidae). *C. R. Acad. Sci. U.R.S.S.* **24**: 616–619.

Fujiwara H. & Ogai S. 2001: Ecdysteroid-induced programmed cell death and cell proliferation during pupal wing development of the silkworm, Bombyx mori. *Dev. Genes Evol*. **211**: 118–123.

Kawasaki H. 1989: Methods for culture for wing discs. *J. Tissue Cult. Methods* **12**: 31–33.

Lobbia S., Niitsu S. & Fujiwara H. 2003: Female-specific wing degeneration caused by ecdysteroid in the Tussock Moth, Orgyia recens: Hormonal and developmental regulation of sexual dimorphism. *J. Insect Sci*. **3**: 1–7.

Lobbia S., Futahashi R. & Fujiwara H. 2007: Modulation of the ecdysteroid-induced cell death by juvenile hormone during pupal wing development of Lepidoptera. *Arch. Insect Biochem. Physiol.* **65**: 152–163.

Nardi J.B. & Willis J.H. 1979: Control of cuticle formation by imaginal discs in vitro. *Dev. Biol*. **68**: 381–395.

Nardi J.B., Godfrey G.L. & Bergtrom R.A. 1991: Programmed cell death in the wings of Orgyia leucostigma. *J. Morphol.* **209**: 121–131.

Niitsu S. 2001: Wing degeneration due to apoptosis in the female of the winter moth, Nyssiodes lefuarius. *Entomol. Sci*. **4**: 1–7.

Niitsu S. 2003: Postembryonic development of the wing imaginal disc in the female wingless bagworm moth, Eumeta variegata (Lepidoptera, Psychidae). *J. Morphol*. **257**: 164–170.

Niitsu S. & Kobayashi Y. 2008: The developmental process during metamorphosis that results in wing reduction in females of three species of wingless-legged bagworm moths, Taleporia trichopterella, Bacotia sakabei and Proutia sp. (Lepidoptera: Psychidae). *Eur. J. Entomol.* **105**: 699–708.

Niitsu S., Lobbia S., Izumi S. & Fujiwara H. 2008: Female-specific wing degeneration is triggered by ecdysteroid in the cultures of wing discs from the bagworm moth, Eumeta variegata (Insecta: Lepidoptera, Psychidae). *Cell Tissue Res.* **333**: 169–173.

Oberlander H., Leach C.E. & Tomblin C. 1973: Cuticle deposition in imaginal disks of three species of Lepidoptera: Effect of ecdysone in vitro. *J. Insect Physiol*. **19**: 993–998.

Riddiford L.M. 1976: Hormonal control of insect epidermal cell commitment in vitro. *Nature* **259**: 115–117.

Received January 29, 2010; revised and accepted April 27, 2010