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Original article

Undetectability of vitamin A in bee brood

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Summary — High performance liquid chromatography (HPLC) of larval and pupal stages of the honey bee *(Apis mellifera)* failed to detect any trace of retinol (vitamin A) or retinyl palmitate contrary to previous reports based on Carr–Price colorimetry. Bee brood is not a source of retinol for dietary or cosmetic purposes.

bee brood / *Apis mellifera* / HPLC / retinol

INTRODUCTION

The presence of vitamin A (retinol) in bee brood *(Apis mellifera)* was first reported in 1960 by Hocking and Matsumara and reaffirmed in 1973 by Ishiguro *et al* who included 3 species of immature wasps *(Vespa).* Both sets of investigators used a technique based on the color response of a sample exposed to antimony trichloride; a classical method (Carr and Price, 1926) which occasionally yields false positive results (Drujan, 1971). Hocking and Matsumara found remarkably high levels of vitamin A in bee brood, ranging from 49 to 119 IU/g wet weight (14.7–35.7 RE or μg/g). These levels have been accepted as correct since that time (*eg,* Gary, 1961; Witherell, 1975).

Using high performance liquid chromatography (HPLC), we have been unable to detect vitamin A in a variety of bee brood samples. We wish to draw attention to this result because of the uncertain role, outside of vision, which vitamin A plays in insect ontogeny and physiology and because there is a large lay literature which advocates dietary and cosmetic use of bee brood for its allegedly high vitamin A content (and other micronutrients) (*eg,* Robson, 1991).

Vitamin A and insect physiology

The significance of vitamin A (or provitamin A carotenoids) in insect physiology is apparently limited to specific functions and species. Insects commonly contain pig-

mentary carotenoids, usually in the oil droplets of fat bodies and eggs (Kayser, 1985) which absorb visible light in the 400–500 nm range and hence appear yellow to red. Most insect carotenoids have a C_{22} central polyene chain which, in earlier studies, was quantified through its participation in the Carr–Price reaction. While most carotenoids originate from dietary plants, some derive from microbial symbionts or from predation (Kayser, 1982a). The egg, larval and adult stages of a variety of stick insects contain carotenoids in the 34–66 µg/g range (Kayser, 1982b). *Schistocerca gregaria* eggs normally contain enough carotene for complete larval development, according to Chapman (1979). Hymenoptera have been little studied for carotenoids, although a variety have been found in bee heads and presumably reflect dietary habits (Underwood, 1984). Cartenoids are broken down most commonly into retinaldehyde, which participates in the visual cycle. A role for vitamin A in the vision of adult insects was first shown for honey bees *(A mellifera)* by Goldsmith and Warner (1964) using the Carr–Price technique. They reported quantities of retinaldehyde, in the head only, ranging up to 0.1 µg/g in light-adapted bees. Even lesser amounts (*ca* 0.02 µg/g) of retinol are reported among single adult male and female honey bees (Pennino *et al,* 1991) and it was concluded that insects are poor dietary sources of vitamin A for insectivores although their requirements may be low (Dierenfeld, 1993). Similar results have been shown among species of Orthoptera, Odonata, Lepidoptera, Coleoptera and Diptera (White, 1985). No vitamin A was found in living termites from French West Africa (Auffret and Tanguy, 1947, 1948, cited in Bodenheimer, 1951).

There is no evidence that vitamin A and β-carotene are essential for basic insect survival. However, a universal role for carotenoid pigments is in protecting cells from photo-oxidative damage. Vitamin A has been shown to promote growth in sarcophagid *Agria affinis* while β-carotene is beneficial in the growth of *Locusta migratoria* (Reinecke, 1985) and larval silkworm *(Bombys mori)* (Shimizu *et al,* 1981). Photosensitivity of a variety of larval insects raised on carotenoid (or vitamin A) free diets declines over time according to Kayser (1982b). Obviously, a phototactic feeding mechanism is adaptive for such insects but is not required in honey bee larvae provisioned by adults.

Ishiguro *et al* (1973) reported average levels of putative vitamin A in immature *A mellifera*, using the Carr–Price technique, that declined from about 1.36 to 0.89 µg/g between larval and imago stages, which they concluded, for reasons that are not quite clear, indicated that its role must be in photosensitivity rather than growth promotion. In their original study, Hocking and Matsumara (1960) did not speculate on the physiological role of vitamin A in the bee.

It is uncertain whether natural larval food in the honey bee contains a lot, a little or no vitamin A (Haydak, 1970). There is an abundance of carotenoids in pollen (Vivino and Palmer, 1944; Winston, 1987). The color of beeswax is due primarily to the β-carotene in the pollen and thus there is no shortage of provitamin A for nurse bees and larvae. Nurse bees require pollen or a pollen substitute to support normal growth and development of larvae (Crailshem, 1990). Older nurse honey bees lose metabolic competence to produce larval food that contains normal vitamin levels (retinol was not examined) (Standifer and Mills, 1977). In a study of fat soluble requirements of worker bees, Herbert and Shimanuki (1978) showed that the greatest response, in terms of larvae raised successfully to the sealed stage, was to vitamin A, but that fat soluble vitamins were not essential in bee diets. The manner in which vitamin A improves

brood rearing is not discussed; presumably its contribution to vision is important.

In conclusion, the role of vitamin A in insect physiology is limited to the vision requirements of young and full adults; there is no need to assume the presence of visual pigment in immature bees. Consequently, it seems unlikely that bee brood would contain significant amounts of vitamin A.

MATERIALS AND METHODS

We employed chromatographic separation using HPLC in a system that has routinely been used by one of the authors for the detection and measurement of retinol in serum samples and biological tissues such as liver (Leichter *et al*, 1991).

Sample collection

Honey bee brood were collected in June 1991 from hives on Burnaby Mountain, British Columbia (courtesy of M Winston, Simon Fraser University). Capped and uncapped brood ($n = 100$) were extracted by suction pipette from frames previously warmed in an incubator or, more usually, chilled in a freezer (–20°C) and placed in groups of 5–10 individuals in 1.5 ml microcentrifuge tubes for collective weighing (wet weight). The samples were frozen in liquid nitrogen and stored in the dark at –80°C for periods ranging from 4 to 24 d.

Extraction

Extraction was performed under > 500 nm fluorescent lighting (gold-colored fluorescent tubes) to prevent sample degradation. Frozen larvae from successive developmental groups (small, medium and large larvae, prepupae and pupae) were allowed to thaw partially on ice (*ca* 1 h). A tissue mass of 200–500 mg plus 9 parts water by weight were liquified at 4°C by homogenization with a motorized mechanical/ultrasonic tissue homogenizer (Polytron, Brinkmann Instruments). From this homogenate, replicate 200 µl aliquots were pipetted into 1.5 ml polypropylene centrifuge tubes containing 200 µl of 1 M KOH in ethanol. The tubes were heated at 50°C for 1 h and periodically shaken to saponify the lipids and digest the tissue so as to release both free and esterified retinol. Following saponification, 800 µl hexane was added to each tube, and the tubes were vortexed vigorously for 1 min. Tubes were then centrifuged briefly to separate the hexane phase; 600 µl of hexane from each sample was removed, placed in a 1.5 ml polypropylene centrifuge tube, and dried in a centrifugal vacuum evaporator (Speed-Vac, Savant Instruments). The resulting oily residue was dissolved in 100 µl of 100% ethanol and 50 µl aliquots were injected for HPLC analysis.

High performance liquid chromatography

We used an HPLC system consisting of a Spectra Physics SP 8700 pump coupled to an injector and a Merck RP-18 reversed phase column (5 µm packing, 4 x 250 mm). The column was eluted at 1.0 ml/min with 95% methanol, 5% 20 mM bis-Tris-propane pH 6.8. For detection we used a Kratos 757 UV absorption detector with a tungsten lamp, set at 325 nm. The analytical method was calibrated by injection of reference standards of retinol which had been prepared by spectrophotometry in methanol using a molar extinction coefficient of 52 500.

RESULTS

Based on Hocking and Matsumara's (1960) estimate of vitamin A in bee brood we anticipated a range of approximately 15 to 35 µg retinol per gram sample. With the sample extraction and HPLC procedures used, this corresponds to approximately 110–270 ng of retinol per HPLC run. Figure 1a shows a typical calibration run in which 39 ng retinol was injected onto the HPLC column. The retinol was eluted as a well-resolved peak with a retention time of approximately 6.5 min. Figure 1b shows a typical bee larvae sample, plotted at 5 times the detector sensitivity as was used for chromatography

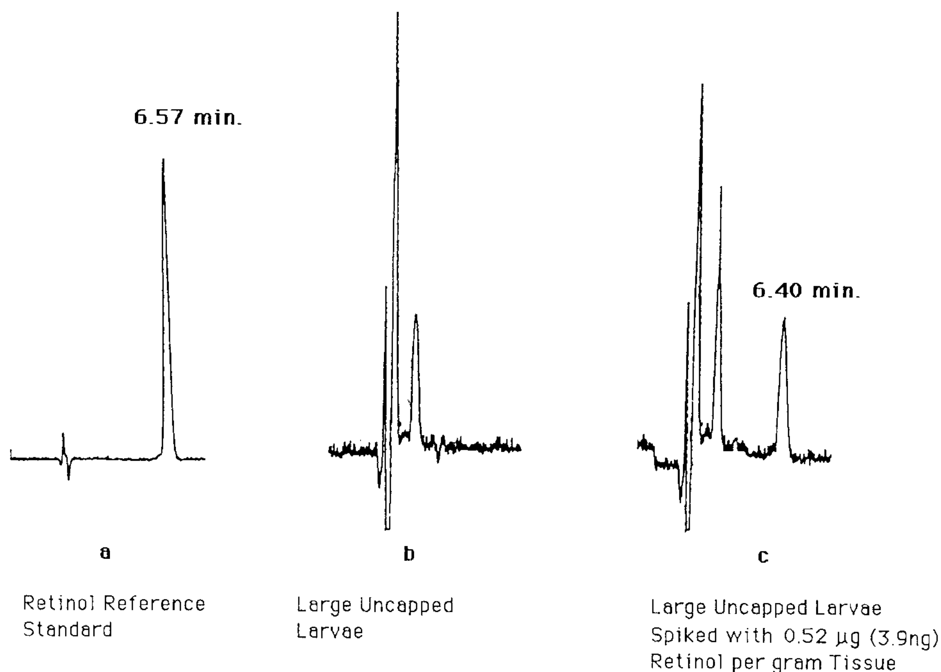

Fig 1. Measurement of retinol by HPLC; (**a**) 39 ng retinol calibration standard; (**b**) typical sample of bee brood (large uncapped larvae) showing no corresponding retinol peak; (**c**) replicate extract of bee brood spiked, after extraction, with retinol equivalent to 0.52 µg/g tissue (3.9 ng). All chromatograms utilize UV detection at 325 nm; chromatogram (**a**) uses a sensitivity of 0.1 absorbance units full scale (AUFS), while chromatograms (**b**) and (**c**) use a sensitivity of 0.01 AUFS.

of the standard. No retinol peak is observed under the conditions used. The detection limit for the method (2:1 signal-to-noise ratio) is approximately 1 ng of retinol per HPLC injection, equivalent to 0.133 µg retinol per gram sample. In a control experiment, extract of bee brood was spiked with retinol equivalent to 0.52 µg/g (3.9 ng) tissue (fig 1c). The spike was added to the extract rather than the homogenate on the assumption that the retinol fraction would be contained in the lipid component. The resulting peak at the retention time of retinol indicates the ability of the method to detect retinol when present. The negative results of assays for retinol were characteristic of all our larval (capped and uncapped) and pupal samples, whether saponified or not. Similar negative results were obtained for analysis of retinol palmitate in unsaponified samples (data not shown).

DISCUSSION

With the instrumentation outlined above, the detectable level for retinol is approximately 100 times lower than the minimum expected values obtained from bee brood by Hocking and Matsumara (1960) using Carr–Price colorimetry. Ishiguru *et al* (1973), who also relied on the Carr–Price technique, reported vitamin A in immature honey bees and wasps, although at levels that were

approximately 10–40 times lower than previously reported. In that our system can detect *ca* 0.1 to 0.2 µg/g of retinol, we think it unlikely that biologically significant amounts of retinol are present in immature *A mellifera*. After emergence, of course, the young worker bee would require carotenoid from pollen to synthesize visual pigment and its head can be expected to contain retinaldehyde (White, 1985).

Some organisms, *eg,* freshwater fish, utilize an alternative visual pigment, with 11-*cis*-3,4-didehydroretinaldehyde, as the chromophore (Morton, 1942). This form, known as vitamin A_2 aldehyde (Chaney, 1960), shows a maximum UV absorption in the range of 340–350 nm. The polarity of vitamin A_2 is very similar to retinol, and therefore would have had a very similar retention time in HPLC. Even at 325 nm (our experimental setting) the dehydroretinol would show some absorbance. We saw no such peak. Furthermore, vitamin A_2 has not been reported in honey bees although the provitamin A_2 carotenoid occurs in stick insects (Kayser, 1982a). For these reasons we do not feel Hocking and Matsumara had inadvertently detected vitamin A_2.

Our research protocol did not test for a third form of visual pigment chromophore, 11-*cis*-3-hydroxyretinaldehyde, which could conceivably account for the Carr–Price positive results found by earlier authors. This form is found in the heads but not bodies of some Diptera and Lepidoptera, but has not been shown, to our knowledge, in *A mellifera* (Goldsmith *et al,* 1986); nor would we expect visual pigments to be present in all but the most mature larvae.

There are several reasons to doubt the presence of vitamin A in bee brood. The brood are unpigmented and hence are unlikely to contain carotenoids. Secondly, the brood larvae do not have visual requirements and hence are unlikely to have retinol. The ommatidia of the eye are not fully developed until the pupal stage (Snodgrass, 1956). A few of the most mature pupae in our sample of 100 immature brood specimens had discernible black eye dots, although these are unlikely to contain visual pigment until after emergence. Thirdly, a role for vitamin A in the growth of most insects has not been substantiated. Therefore, it is important to consider why various workers have obtained positive results using the Carr–Price technique applied to bee brood. A bright orange fungus, *Wallemia sebi,* has been reported in a beehive (Weedon, 1973); however this substance is not a carotenoid and we are unaware if a color reaction would occur in the presence of antimony trichloide. Carr–Price colorimetry was shown as early as 1928 to correlate very closely with UV absorption by fish oils (Morton, 1942) and hence seemed a robust and simple method. However, it is difficult to measure the transient blue color response of retinol/carotenoid to antimony trichloride with accuracy and there is a lack of consistent results using this technique due to its sensitivity to turbidity from even minute traces of moisture (Neeld and Pearson, 1963; Frolick and Olson, 1984). Neither Hocking and Matsumara nor Ishiguru *et al* clarify the means by which the color response was calibrated (Hanson, 1973). Most importantly, the method is not specific for retinol in that a color response can occur in the presence of carotenoids and other unrelated polyene compounds such as fatty acids with conjugated carbon double bonds (Drujan, 1971). Anhydrovitamin A forms in fish-liver oils which have been 'maltreated'; this substance has no biological activity but gives absorption bands in the Carr–Price reaction that overlap those of vitamin A (Barker, 1982). Since the state of the antimony trichloride reagent, temperature and color-producing compounds (other polyenes) can influence the reproducibility of the method, Drujan (1971) recommends that an internal standard be run with each sample. Apparently this was not done by these earlier workers.

A recent publication by one of us (Skinner, 1991) claiming to account for hypervitaminosis A in a *Homo erectus* skeleton from East Africa (ER 1805) dated at 1.5 million years ago (Walker *et al,* 1982; Leakey and Walker, 1985) from consumption of bee brood is clearly negated by our results.

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Résumé — Absence de vitamine A dans le couvain d'abeille. En utilisant le test colorimétrique classique de Carr–Price pour la détection de la vitamine A, certains chercheurs avaient trouvé une concentration très élevée de rétinol dans le couvain d'abeille, si bien que les œufs et les larves d'*Apis mellifera* sont généralement considérés comme une importante source de vitamine A en diététique et en cosmétologie. Du couvain operculé et non operculé (n = 100) provenant de ruches des montagnes de Burnaby (Colombie britannique, Canada) a été prélevé des alvéoles par succion au moyen d'une pipette, et stocké à l'obscurité à –80°C. Plusieurs séries d'extractions ont été réalisées à partir de stades différents de couvain (larves petites à grosses, pré-nymphes et nymphes). Deux cent microlitres d'extrait contenant 200–500 mg de couvain dans 9 parties d'eau ont été saponifiés dans 200 µl de KOH 1 M. Cinquante microlitres de l'homogénat saponifié ont été traités par l'hexane et l'éthanol, puis injectés dans un chromatographe liquide Spectra Physics SP 8700. La détection a été réalisée au moyen d'un détecteur Kratos 757, Le seuil de détection était d'environ 1 ng de rétinol par injection. Nos résultats n'ont montré aucun pic de rétinol dans les conditions de l'expérience. Des résultats négatifs ont également été obtenus lors de la recherche de palmitate de rétinol dans des échantillons non saponifiés. Nous en concluons que les publications antérieures portant sur la présence de vitamine A dans le couvain d'abeille étaient basées sur des résultats faussement positifs dus à l'utilisation de la méthode de Carr–Price, que le couvain d'abeille ne contient pas de vitamine A et n'est donc pas une source de rétinol en diététique et en cosmétologie.

couvain / *Apis mellifera* / HPLC / rétinol

Zusammenfassung — Vitamin A ist in Bienenbrut nicht nachweisbar. Frühere Untersuchungen hatten mit der klassischen kalorimetrischen Nachweismethode für Vitamin A nach Carr–Price bemerkenswert hohe Konzentrationen von Retinol in der Bienenbrut ergeben. Aus diesem Grund werden Eier und Larven von *Apis mellifera* allgemein als wichtige Quelle für Vitamin A sowohl bei Diäten als auch in der Kosmetik angesehen.

Aus Völkern in den Burnaby Mountains in British Columbia, Kanada, wurde die Brut aus verdeckelten und unverdeckelten Brutzellen (*n* = 100) mit einer Pipette abgesaugt und bei –80°C dunkel aufbewahrt. Aus aufeinanderfolgender Entwicklungsstadien (kleine bis große Larven, Vorpuppen und Puppen) wurden wiederholt gleiche Proben (200 µl) aus 200–500 mg Gewebe mit 9 Teilen Wasser versetzt und mit 1 mol KOH (200 µl) verseift. Jeweils 50 µl dieses verseiften Homogenats wurden mit Hexan ausgeschüttelt, zentrifugiert und die Hexanphase eingeengt. Der ölige Rest wurde in Ethanol gelöst und in einen Hochleistungs – Flüssigchromatographen (HPLC) injiziert. Dieser bestand aus einer Spectra-Physics SP 8700 Pumpe, einem Injektor und einer Merck RP-18 *'reversed-phase'* Säule (Packung 5 Mikron, 4 mm x 250 mm). Die

Elution erfolgte mit 95% Methanol und 5% 20 mM Bis-Tris-Propan pH 6,8 bei einer Fließgeschwindigkeit von 1,0 ml/min. Der Nachweis erfolgte bei 325 nm in einem Kratos 757 Absorptionsdetektor mit einer Tungsten Lampe. Die Nachweisgrenze dieser Methode beträgt ungefähr 1 ng Retinol pro Injektion. In keiner der Brutproben konnte Retinol nachgewiesen werden (Abb 1b). Eine Probe des Homogenisats wurde mit 3,5 ng Retinol versetzt, was einer Konzentration von 0,52 µg/g Gewebe entspricht. Diese Probe zeigte einen Peak in der richtigen Retentionszeit (Abb 1c).

Bei Anwendung dieser HPLC-Methode konnte kein Retinol-Peak in der Brut nachgewiesen werden. Ähnliche Ergebnisse wurden bei der Analyse von Retinolpalmitat in unverseiften Proben erzielt. Wir schließen daraus, daß die früheren Berichte über Vitamin A in Bienenbrut auf falschen positiven Ergebnissen, bedingt durch die damals verwendete Carr-Price Technik, beruhen. Bienenbrut enthält damit kein Vitamin A und stellt keine bedeutende Quelle von Retinol für Diäten oder Kosmetik dar.

***Apis mellifera* / Retinol / HPLC-Methode / Bienenbrut**

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