A Comparative Study of the Endocrine System of the Honey Bee Larvae under Normal and Experimental Conditions

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The endocrine system of the honey bee (*Apis mellifera* L.) has been studied morphologically through post-embryonic development with several histological techniques. Marked differences in the structure of the neurosecretory complex of queen and worker larvae have been observed during larval stages. In queen larvae, morphogenesis of the neurosecretory cells, their axons and the formation of the chiasma takes place during end of 2nd and beginning of 3rd, in the workers at beginning of 4th larval instar. Stainable neurosecretory material was found in queen larvae at the beginning, in worker larvae at the end of 4th instar. In early larval stages, the corpora allata are more active in the queen. During initial 3—5 days of larval development the gland volume is reduced in both castes. After 36 to 48 hours of endocrine retardation, the glands become active again. The same histological effects are found under experimental conditions, where worker larvae of 2nd instar were reared in the incubator on basic food, Royal Jelly and with topically applied juvenile hormone I.

The female honey bee larva can develop into two phenotypically different forms, the queen and the worker. Differential nutrition has been reported to cause hormonal differentiation which in turn characterises metabolic differences and thus ends up with the dichotomous forms (for review see [1-6]). The determining principle lies in the Royal Jelly because a 2 day worker larva fed on Royal Jelly can develop into a queen or an intercaste [7]. The function of the queen bee determining principle (determinator) has been explained by Rembold [8] as to overcome a hormonal deficiency during development of the female honey bee larva. Therefore, in order to study the mode of action of nutritional factors it becomes necessary to gain more knowledge about the endocrine system of the honey bee larval types during caste determination. Like any other insect, the endocrine system of the honey bee larva consists of the neurosecretory cells, corpus allatum (CA) and prothoracic glands; the corpus cardiacum develops later at the end of the larval period.

The present investigation was aimed to study: 1. sequential changes in the histological structure of the neurosecretory system and the corpus allatum through postembryonic development in the worker and queen larvae, and 2: the effect of Royal Jelly, basic food and JH I on the endocrine system of the honey bee larva reared under *in vitro* conditions.

Material and Methods

Larvae of the Carnica bee, *Apis mellifera*, from the field hives and the winter room were used. In the latter case the animals were maintained at $25 \pm 1\,°C$; $45 \pm 5\%$ RH and 15 hours light [9]. Age of the larvae was defined according to the following procedures.

(1) For deposition of eggs, the queen was kept on an empty brood frame for 4 h. The frame was then covered with a wire-grate which allowed only the workers (nurse bees) to feed and excluded the queen. By that way, eggs and larvae of a very homogeneous age could be obtained.

(2) According to Wang [10], the body weight of brood larvae was used as a measure of age.

(3) Freshly emerged larvae were reared under *in vitro* conditions [9]. Every 3rd hour each individual was observed for timing of its molting. The data obtained under these *in vitro* conditions were similar to those of Bertholf [11] after observation of the brood *in vivo* in the hive (Table I).

(4) Pupal stages were defined as described earlier [12].

For *in vitro* experiments, worker larvae in 2nd instar were kept in an incubator at 35 °C and 85% RH [9]. Six *in vitro* experiments were set up with different food treatments, each consisted of 30 − 40 larvae. Five larvae from each treatment were removed each day and used for histological examina-

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tion of the endocrine tissues. For control of determining activity, 60 larvae were reared in tests I – VI (Table II) completely to adults. In experiment I, the larvae were reared on basic food. In experiment II, the determinator fraction from Royal Jelly [7, 9] was added to basic food and in experiment III, the larvae were reared on Royal Jelly,

Table I. Time sequence of instars and moults in the honey bee castes according to Berthold [11].

days	hours	Queen		Worker	
		instars	ecdyses	instars	ecdyses
1					
2		egg		egg	
3	0				
4	18	1st	(hatching) 1st	1st	(hatching) 1st
5	36	2nd	2nd	2nd	2nd
5	60	3rd		3rd	
6	84	4th	3rd	4th	3rd
7			4th		4th
8		5th		5th	
9	144				
10	168	pharate pupa	5th	pharate pupa	
11	192				5th
12		pupa + pharate adult			
13					
14				pupa + pharate adult	
15			6th (emerging)		
16					
17		imago			
18					
19					
20					6th (emerging)
21				imago	
22					

diluted 1 : 1 with a solution of each 125 mg glucose and fructose per ml water. The composition of basic food has already been reported earlier by Rembold et al. [9]. In experiments IV, V and VI, 1 μg of JH I (Biojine 100, gift from Dr. J. Lhoste, Procida S.A., Puteaux, France, concentration 1 μg/μl) in acetone was topically applied to the 3 days old larvae reared on basic food (IV), basic food + determinator fraction (V), and Royal Jelly (VI).

For histological examination, the head and first thoracal segment were cut off and fixed according to Bouin, Carnoy or Helly [13]. The material was embedded into paraplast. Serial transverse sections were cut at 5 – 8 μm and stained with either (1) aldehyde-fuchsin (AF) [14], counterstain Halmi's mixture, (2) paraldehyde-fuchsin (PAF) [15], counterstain light green, (3) aldehyde-thionin (AT) [16].

Results

1. Normal conditions

Neurosecretory cells and axons

During the early larval period, the neurosecretory complex is built up and becomes active in the 4th larval instar. In the first and second instar, a group of cells develops in the pars intercerebralis which is distinctly separated from the motor neurons (Fig. 1-1). Later on, the neurosecretory cells differentiate from these cells. As a characteristic, mitotic stages are found in this area. The neurosecretory axons grow faster in the queen larva than in the worker of same instar (Fig. 1-2, 1-3). A distinct chiasma becomes visible in the queen at the end of 2nd to beginning of 3rd instar and in the worker at the beginning of 4th instar (Fig. 1-4). Stainable neurosecretory material could be found in this area only in the larvae of 4th instar. In queen larvae it appeared a few hours after moulting and in the workers at the end of this period. This lag in worker neurosecretory material production corresponds to a difference of about one day (Fig. 1-5).

Corpora allata and corpus cardiacum

During first and second instars (L1 and L2), size and structure of the corpora allata are very similar in both the castes with an average diameter of 40 μm (Fig. 2-1). A caste specific morphological difference can be seen only with the third larval instar.

During L3, the volume of queen CA increases faster than that of the worker larva. At the end of this instar, CA of the queen measures about 120 μm and that of the worker about 80 μm in diameter. In the queen CA, chromatin is condensed during the first 18 to 20 hours of third larval instar. The cell shape can distinctly be identified (Fig. 2-2). Then chromosomes gradually become decondensed and evenly distributed. The cell borders disappear concomitantly (Fig. 2-3, 2-4). The same histological picture is observed during fourth larval instar of the queen caste.

As well as in L3 queen larva, chromosomes are condensed and closely packed in the cell nuclei of the worker CA. In contrast to the queen, the histological picture of CA of the worker is not changed during the whole period of third instar (Fig. 2-6, 2-7). Decondensation of chromosomes and disappearance of cell borders is delayed till middle of
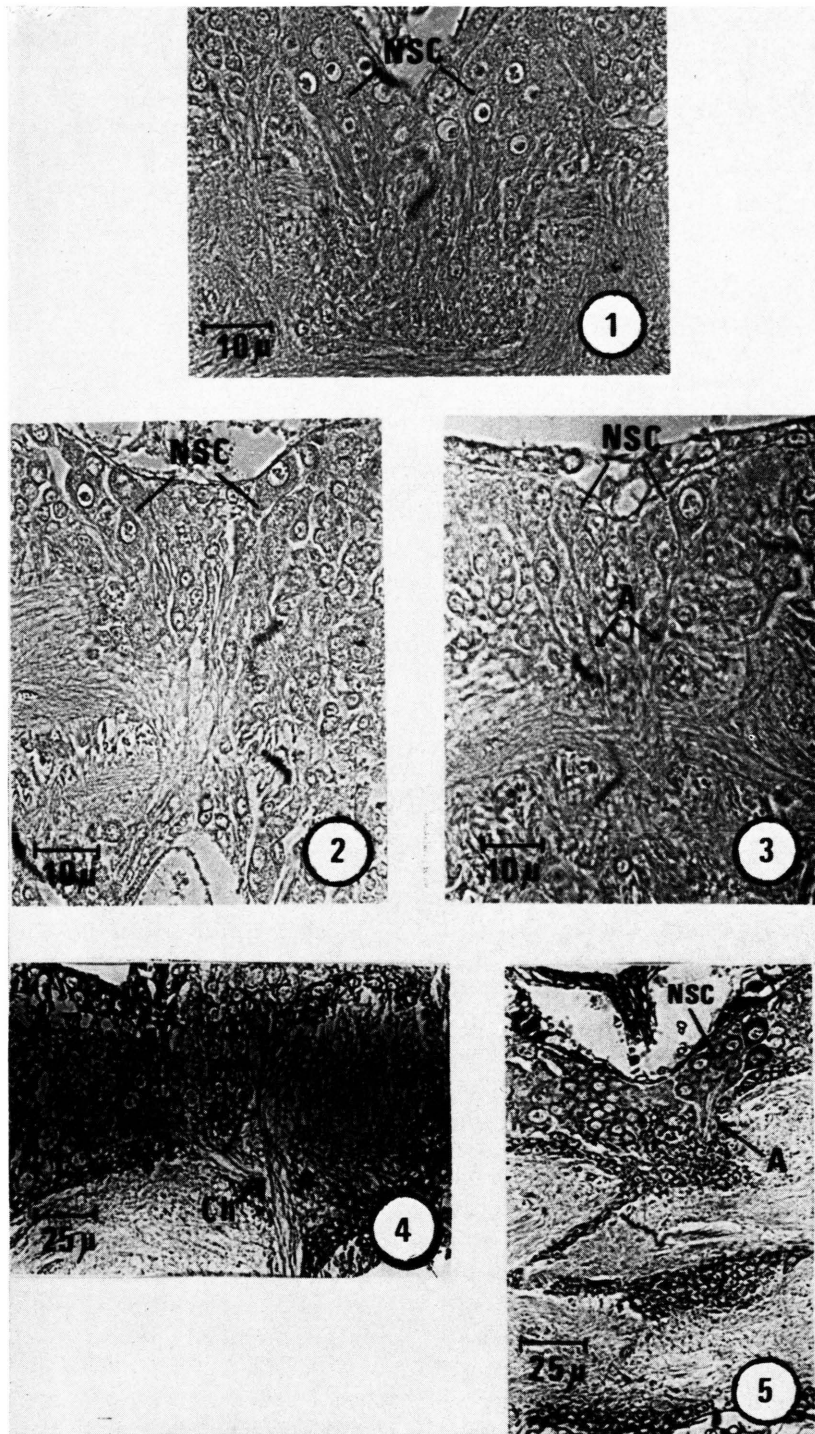
Fig. 1. Changes in histological shape of pars intercerebralis during larval development of honey bee castes. 1-1. Pars inter-cerebralis of a worker larva, stage L2; 1-2. Pars intercerebralis of a worker larva, stage L3; 1-3. Pars intercerebralis of a queen larva, stage L3; 1-4. Chiasma of neural axons in a queen larva, stage L3; 1-5. Pars intercerebralis containing cells with stained neurosecretory granules in a queen larva, stage L4; Abbreviations: NSC = neurosecretory cells, A = axons, Ch = chiasma.
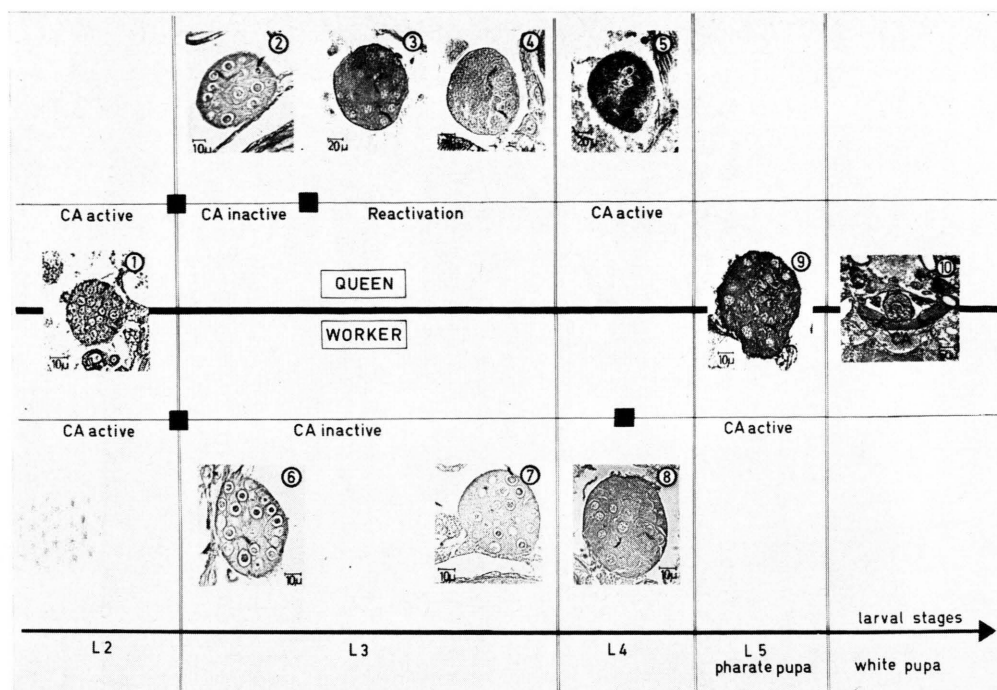
Fig. 2. Sequence of cellular modulation of corpus allatum activity during larval development of queen and worker castes of the honey bee.

fourth instar corresponding to a time difference of about one day versus the queen (Fig. 2-8). During fifth larval and the pupal stages, the corpora allata of both the castes have an identical appearence again (Fig. 2-9, 2-10). Despite the fact that stainable neurosecretory material is not visible in the neurosecretory cells and tract until 4th instar, enough material was observed in the axons inside the corpus allatum of both the castes as well under normal as under experimental conditions. The corpus cardiacum is represented only by a single layer of cells in the wall of the aorta. The gland develops later in the five days old larvae in the queen and at white pupal stage in the workers. There is a gradual built up of material in the gland during metamorphosis so that in the pharate adult the corpus cardiacum is much loaded with neurosecretory material (NSM).

These data clearly demonstrate that the endocrine complex of the queen caste develops faster as well from a morphological as from a functional point of view. The phase of retarded corpus allatum activity ends earlier in the queen and is correlated in both the castes with the beginning of production of stainable neurosecretory material in the pars inter-

cerebralis between end of 3rd and beginning of 4th larval instar.

2. Experimental conditions

In all the experiments the first batch of larvae was fixed in the 3rd instar, *i. e.*, after 24 hours under *in vitro* conditions. In these larvae from all the six different food treatments, an endocrine retardation of the type described above was observed. In the corpora allata the nucleoplasmic ratio is similar to that under normal conditions.

The response of the worker larvae to varying food conditions is significant (Table II). On basic food, only one from 53 adults developed to a queen, 10 to intercastes (experiment I). As well after addition of the active fraction to basic food (experiment II), as with native Royal Jelly (experiment III), considerably more of the adults have become queens. Topical application of juvenile hormone I stimulates development of intercastes primarily (experiments IV – VI). The histological picture corresponds to these data: here, as with queens under normal conditions, larvae from tests with high queen determining activity (experiments II, III and V) exhibit an early termination of re-

Table II. Percentage of differentiation (%) to queens and intercastes after rearing of 60 two day old honey bee worker larvae under different experimental conditions. For further details see Material and Methods.

Exp. No.	Experimental conditions	Adults (n)	Queens	Intercastes	Workers	%
I	basic food	53	1	10	42	21
II	basic food + determinator fraction	49	12	9	28	43
III	native Royal Jelly	54	17	8	29	48
IV	basic food + juvenile hormone I 1 μg topically applied	49	9	16	24	51
V	basic food + determinator fraction + juvenile hormone I, 1 μg topically applied	46	16	17	13	71
VI	native Royal Jelly + juvenile hormone I, 1 μg topically applied	57	2	23	32	44

tarded corpus allatum activity and a concomitant growth of axons and chiasma formation, whereas larvae from basic food exhibit a similar histological picture as worker larvae from the colony. The same holds true for the experiments with juvenile hormone (experiments IV – VI). Corresponding to the high degree of intercastes, the end of corpus allatum retardation varies considerably, speaking for a tight correlation of corpus allatum reactivation and queen bee establishment.

Discussion

Our results suggest that there is a significant difference in the developmental pattern of the endocrine system of the queen and worker larvae. The neurosecretory cells in the queen grow faster than those of the workers and a chiasma of the axons is formed here between the end of second and beginning of third instar of larval life. In workers, it is developed with the beginning of 4th larval instar. This time difference in development corresponds to an average of one day and is coupled with the beginning of a pronounced increase in body weight in the queen larva in the third instar. Our results agree with those of Formigoni [17] who reported that a 3 day larva contains neurosecretory cells, but differ from those of Canetti et al. [18] who did not observe neurosecretory cells with axons until pupal period. Later on, Ritcey and Dixon [19] observed neurosecretory cells in the third larval instars of both the castes. The significant difference in the corpus allatum volume and the nucleoplasmic ratio during the first three days reflects the net difference in the

synthetic activity of the gland of the two castes. The large gland of the queen larva seems to be more active than that of the worker. The differences at the ultrastructural level and the quantitative differences in the haemolymph JH concentration reported by Wirtz [20] supports our histological findings.

The most significant feature of our results is a temporary retardation of endocrine activity in both the castes. This ends with a caste specific reactivation of corpus allatum activity. The cause for this is not clear from our static histological observations. It is known that at about 3 to $3^1/_2$ days of larval life, there is a change in the food of worker larvae from worker jelly to mixed worker jelly [21]. A similar change has been reported by von Rhein [22] for the queen larva. It could be that the change in the food conditions of the two larval types at about the same time is responsible for the caste specific events. Such an interpretation receives support from our results with in vitro experiments.

The available information on the structure and functional significance of the corpus allatum is rather controversial and does not permit a conclusive inference on the role of this gland in the development of the polymorphic forms. Pflugfelder [23], Lukoschus [24], and Canetti et al. [18] reported a progressive increase in corpus allatum volume in both the larval types which is comparatively much more in the queen larva. A nuclear degeneration in the CA of the queen larva was observed between the 3rd and 4th instars in contrast to worker larvae [18]. Wirtz [20] reported that topical application of JH in vivo to 3 days old worker larvae results in the development of queenlike adults. Rembold et al. [25] did not notice an effect of JH I in queen forma-

tion, but only an increase in the formation of intercastes in the larvae reared *in vitro*.

An interesting feature of our *in vitro* experiments is the varied response of the endocrine tissues to different food treatments. In the experiment with basic food + determinator (experiment II) and with Royal Jelly (experiment III), an early end of endocrine retardation in the 4th instar suggests that a factor from Royal Jelly is responsible. Many of these larvae renew their activity in the early 4th instar and these must be the larvae which develop into queens. A delayed response of the endocrine tissues results in their development into intercastes and workers. The effect of JH I application is not as distinct from our histological results. Due to a high yield in intercastes, formation of a chiasma and the end of endocrine retardation in the corpora allata varies in between the caste specific behaviour of queen and worker larvae, respectively.

Finally, it could be speculated from these results that the endocrine retardation corresponds to a hormond deficiency in juvenile hormone synthesis primarily and that this effect is genetically fixed. This labile endocrine situation must be ended by an external event which obviously is identical with a difference in food quality for both the castes. As a first caste specific event, growth of axons from the neurosecretory cells and formation of a chiasma starts in the prospective queens with a subsequent appearance of stainable colloids in the corpora allata and followed by an intensive growth. The same sequence is followed in the prospective worker with a lag time of about one day. This is the first possibility to shift into a caste specific development under endocrine control in the female honey bee larva.

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