Original article

Characterization of preimaginal developmental stages in Africanized honey bee workers (*Apis mellifera* L)

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Summary — The stages of preimaginal development of the Africanized worker honey bee (*Apis mellifera* L) were characterized. Body weight and head diameter were used as characters with which to differentiate among the first 4 instars, and additional traits such as apolysis, defecation and tibiotarsal length of the hind legs were used to characterize the subdivision of the 5th and last instar. Pupal stages were determined on the basis of eye and thorax pigmentation. The duration of each stage was also determined from these characteristics. The post-embryonic development period of Africanized bees is ≈ 24 h shorter than that in *Apis mellifera carnica*, and 18 and 24 h longer than in African bees.

Africanized honeybee / preimaginal development / duration of development / larval instar

INTRODUCTION

Like other holometabolic insects, honey bees present 3 different phases (larva, pupa and adult), separated by metamorphic moults. The larval phase is mainly a period of feeding and growth. The end of this phase is marked by the beginning of metamorphosis, which consists of a complex reorganization of larval structures and the formation of imaginal structures. After pupal ecdysis, the pupa undergoes a series of changes in the pigmentation of the compound eyes and of the body. The pupal/imago moult occurs at the end of this phase.

Several investigators have described the preimaginal development of *Apis mellifera* L using European honey bees (Nelson, 1924; Bertholf, 1925; Myser, 1954; Rembold *et al*, 1980; Thrasyvoulou and Benton, 1982). The presence of 5 larval instars has been determined by these studies and the final larval instar has been subdivided according to the dramatic changes occurring in it (Rembold *et al*, 1980; Rachinsky, 1990). Several stages have also been observed in the pupal phase.

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However, the duration of these stages varies among different bee races such as the Europeans, Africans and their hybrids (Africanized) (Kerr et al, 1970; Garofalo, 1977; Tribe and Fletcher, 1977; Fletcher, 1978; Palacio, 1991). When the duration of the post-embryonic development stages of Africanized and European bees were compared in a common environment, differences were also found (Rosenkranz, 1990; Palacio, 1991). In contrast, Morini (1990) observed similar duration of post-embryonic stages in a study comparing European and Africanized honey bees.

Nevertheless, a detailed characterization of the developmental phases has not been available for Africanized bees. Thus, the objective of the present investigation was to provide a detailed profile of the pre-imaginal development of Africanized bees using morphological and physiological characters, in order to facilitate studies in different areas in which the identification and duration of these stages are important requirements.

MATERIALS AND METHODS

The study was conducted with Africanized honey bees from the Experimental Apiary of the Department of Genetics, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, SP, Brazil (21°11'25" latitude S and 47°43'04" longitude W). The bees were maintained in Langstroth-type hives with 10 frames containing a standardized number of bees. During periods of food scarcity, colonies were fed sucrose syrup (60% w/v).

In order to obtain controlled oviposition, the queen of a colony was secured to a comb with the aid of an excluder cage for a period of 6 h. In order to reduce the disturbance to the colony during sample collection, a group of 5 colonies was used alternately. Samples were taken from each colony a maximum of once a day. Eggs were monitored beginning 60 h later in order to determine the precise moment of hatching. The brood combs were maintained in their original

colonies and samples were collected at 6-h intervals from hatching to imago. During the larval phase, larvae were weighed and their head diameter (measured at the antennal imaginal discs) was measured using a stereomicroscope. The larvae collected before cell sealing were washed in physiological saline to remove any traces of food remaining on their bodies. During the 5th and last larval instar, the position and movement of the larva within the cell, apolysis, defecation and tibio-tarsal length of the hind legs, in addition to weight and head diameter, were used to characterize the subdivisions of this instar. The leg primordia are evident beginning at the 3rd larval instar with their respective segments (Myser, 1954). At the 5th larval instar (after cell sealing), the tibia and tarsus of the 3rd pair of legs were measured through dissection of the leg buds in physiological saline. In the prepupa these can be measured through the transparent larval cuticle. A total of 1 249 larvae were analyzed. For the determination of pupal stages, the brood comb was left in the hive until the prepupal stage, when samples started to be collected at 6-h intervals. Eye colour and thorax pigmentation were classified using the international color standard of the Munsell color book (Macbeth Division of Kollmorgen Corp, Baltimore, MD, 1976).

The general conditions of the colonies were carefully maintained throughout the characterization of larval and pupal stages in order to avoid any interference with the development of larvae and pupae. Specimens were collected during various seasons in order to minimize possible environmental effects.

RESULTS

Characterization of larval instars

When a comparison was made between the log of the body weight and the diameter of the head capsule of 1 249 worker larvae 5 groups become evident (fig 1), each corresponding to a larval stage. The intervals between groups represent the larval ecdyses, as determined by Brook–Dyar's rule (Hutchinson and Tongring, 1984). Ac-
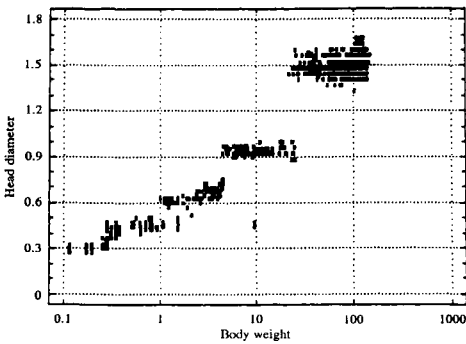
Fig 1. Correlation between body weight and head diameter. R^2 = 0.7585 highly significant; $P < 0.01$.

cording to this rule, the number of disconti-nuities in a sclerotized non-articulating structure, as determined by measure-ments, corresponds to the number of larval instars.

Body weight markedly increases (a sig-moid curve) during the first 4 larval instars, up to the moment of sealing the larval cell (fig 2), when it decreases slightly just be-fore the pupal ecdysis. The weight ranges and mean head diameters observed in the

5 larval instars are presented in table I, with a significant difference between the mean head diameter values ($P < 0.01$). The 5th and longest larval instar can be subdivided into 9 periods which are marked by clearly identifiable events that distinguish several subdivisions or succes-sive phases of the 5th larval stage. These are: 3 involving the end of the feeding phase (LF1, LF2 and LF3); 3 involving the cocoon-spinning phase (LS1, LS2 and LS3), and the last 3 correspond to the pre-pupal phase (PP1, PP2 and PP3). Since no easily recognizable outer changes oc-cur during the feeding periods, the change in body weight was used as a parameter for the identification of these periods. The first subdivision (LF1) corresponds to the beginning of the last period of larval feed-ing after the moult to the 5th instar, with larvae weighing an average of 34.87 ± 7.75 mg. Larvae weigh an average of 72.05 ± 18.96 mg during LF2, whereas at the end of these instar, during LS3, aver-age larval weight is 111.32 ± 4.54 mg. The mean larval weight during the different pe-riods of the 5th larval instar and the prepu-
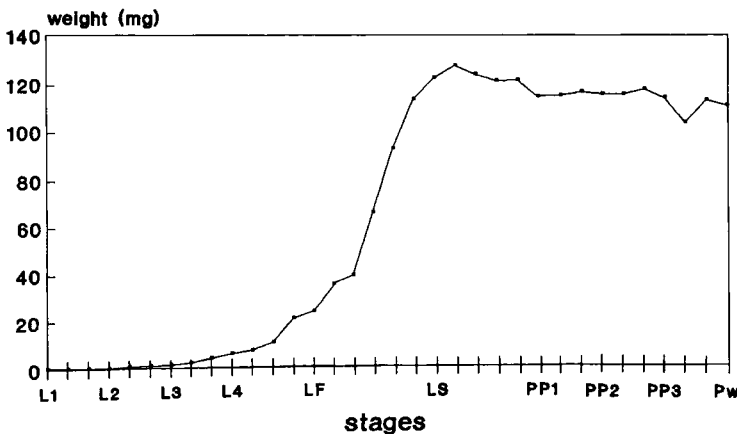

Fig 2. Mean weight of worker larvae from the first larval instar (L1) to the white-eyed pupal stage (Pw).

Table I. Weight range and mean head diameters during the larval instars.

Instar[a]	Weight (mg) (min-max)	n	Mean head diameter (mm)	SD[b]	n
L1	0.11–0.30	139	0.32	0.0256	57
L2	0.31–1.05	135	0.44	0.0321	53
L3	1.50–4.45	66	0.65	0.0451	59
L4	4.80–24.8	59	0.92	0.0939	117
L5	24.30–126.7	354	1.49	0.0475	121

[a] The larval instars are symbolized as follows: L1–L5 = 1st–5th larval instar; [b] SD: standard deviation.

Table II. Mean weights during the periods of the 5th larval instar.

Periods *	n	Weight (mg)	SD
LF1	121	34.87 [h]	7.752
LF2	176	72.05 [g]	18.960
LF3	24	111.32 [cde]	4.540
LS1	154	124.21 [a]	7.483
LS2/LS3	47	117.88 [b]	4.650
PP1	130	113.64 [cd]	6.346
PP2	105	112.57 [d]	4.755
PP3	53	107.55 [f]	5.909

* LF1–LF3 = last larval periods; LS1–LS3 = larval periods after sealing; PP1–PP3 = prepupal periods; 2) [a, b, c, d, e, f, g, h] different letters in superscript indicat a statistical difference ($P < 0.01$).

pal period are presented in table II. The differences between these groups were significant ($P < 0.01$), except for LF3, PP1 and PP2. At the end of the feeding periods, the brood cells are sealed by the nurse bees. Approximately 2 h later, the larva starts spinning its cocoon (Rachinsky, 1990). Since cocoon spinning is difficult to detect, we considered cell sealing as one of the parameters for the determination of this new period (LS) during which

the larva reaches its maximum weight. The additional parameters used were: defecation, larval position within the cell, and the length of the tibia and tarsus of the third pair of legs. At LS1, the larva is rolled up inside the cell and moves about. The intestine is completely filled with a yellowish materal; the tibio-tarsal length is 0.8 to 1.0 mm, and does not change until the end of LS3. Defecation marks LS2, during which one-half the length of the intestine empties. At LS3, defecation terminates. The larva then extends lengthwise in the sealed cell with its head oriented toward the operculum, and becomes motionless. This marks the beginning of the prepupal period. The intestine is completely empty but is still visible in the dorsal region of the larva. The prepupa is characterized by profound morphological modifications such as the enlargement of the eyes. The antennae remain S-shaped but are longer and distinctly 12-segmented. The thorax is separated from the head by a distinct constriction. An easily identifiable event is apolysis, which begins each moulting cycle (Hinton, 1973), but is more easily recognized during this period. For this reason, the progression of apolysis from the cephalic to the posterior region was also utilized to characterize the prepupal subdivisions. Apolysis begins in the head region

and is observable as a separation of the larval cuticle from the newly forming cuticle, with the space between cuticles filled with exuvial fluid. During the 1st prepupal period (PP1), the intestine can be visualized as an empty tube, and the tibio-tarsal length is 1.20 to 1.44 mm. At PP2, the intestine is no longer visible, and the tibio-tarsal length increases to 1.80 to 2.00 mm. When apolysis extends to the thorax and abdomen, the appendages of the head and thorax are visible through the cuticle. At the end of the prepupal stage (PP3), the exuvial fluid is reabsorbed, and the digested larval cuticle become opaque and wrinkled. The thoracic appendages are no longer visible through the larval cuticle. Ecdysis is the next step towards completion of metamorphosis.

Characterization of pupal stages

Seven pupal stages were identified during the pupal phase according to eye and thorax colour (table III).

Table III. Characterization of pupal stages.

Stage	Pigmentation of eye	Pigmentation of thorax (dorsal mesothorax)
Pw	White (5R 9/2)*	–
Pp	Pink (10R 8/4 to 10R 5/4)	–
Pdp	Dark pink (10R 4/4)	–
Pb	Brown (7.5R 3/4)	–
Pbl	" "	Light (2.5Y 8/4)
Pbm	" "	Medium (10YR 7/6 to 10 YR 5/4)
Pbd	" (7.5R 2.5/2)	Dark (10YR 4/2)

* According to the Munsell Color Book. Pw = white-eyed pupae; Pp = pink-eyed pupae; Pdp = dark pink-eyed pupae; Pb = brown-eyed pupae; Pbl = brown-eyed, light pigmented thorax pupae; Pbm = brown-eyed, medium pigmented thorax pupae; Pbd = brown-eyed, dark pigmented thorax pupae.
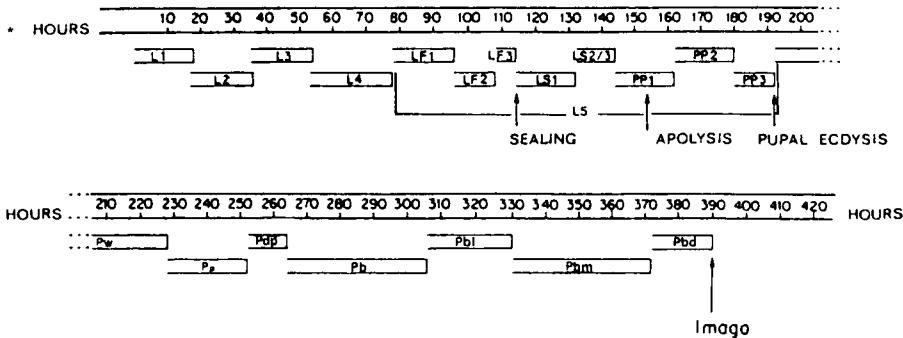

Fig 3. Duration of the stages and periods of preimaginal development, and indication of some events used to characterize these stages. * : hours after hatching.

Duration of larval and pupal stages

The duration of the different stages and periods of preimaginal development determined on the basis of the present data are presented in figure 3. A variation of ± 6 h as well as an overlapping of some stages was observed.

DISCUSSION

According to Thrasyvoulou and Benton (1982), the growth curve of worker larvae gives a good indication that larval weight can be used as an indicator of larval age. This agrees with our results as well those of Wang (1965), Rembold *et al* (1980) and Rachinsky (1990) in European bees. Worker weight increases more rapidly during the first 90 h of development, and queen weight can vary more than worker weight (Wang, 1965). The decrease in larval weight observed here after sealing has also been reported by others in European bees (Jay, 1962; Rembold *et al,* 1980; Rachinsky, 1990). Weight continues to decrease gradually up to adult emergence (Jay, 1961; cited by Jay, 1963).

The first 4 larval instars can be determined in a relatively simple manner on the basis of head size and body weight. By comparing the present results with those reported by Goitein (1989) for Africanized honey bee, mean head size was found to be similar for each instar, but the mean duration was found to be longer in his material. However, our results are similar to those of Rosenkranz (1990) and Palacio (1991), with respect to the larval development in Africanized bees. Little variation in larval instar duration was found when we compared our results with those obtained for *A m carnica* (Rembold *et al,* 1980). In contrast, the LF3 period for the 5th larval instar, corresponding to the time of seal-ing, is ≈ 6 h shorter in Africanized bees than in *A m carnica* (Rachinsky, 1990). Another difference is in the prepupal periods, which is 6 h shorter in the bees studied here. These results agree with those of Rosenkranz (1990), who found that the development period from egg laying until cell sealing was shorter in Africanized bees than in *A m carnica* when compared side by side. However, when this difference occurred has not been determined.

The characterization of the different pupal stages presented here is similar to that reported by Jay (1962) and Rembold *et al* (1980). However, as also observed in the larval phase, the duration of these stages differs slightly when we compare our data with those reported for *A m carnica* (Rembold *et al,* 1980). The major differences reside in the white-eyed pupal stage (Pw), which is ≈ 36 h for Africanized bees and 42 h for *A m carnica* (Rembold *et al,* 1980), and in the brown-eyed pupal stage (Pb) which lasts 42 and 48 h in the 2 subtypes, respectively. Thus, the total duration of the preimaginal stages is 24 h shorter in Africanized honey bees than in *A m carnica*, with a variation of 6 h. Therefore, according to these results, as well as those of others authors (Garofalo, 1977; Couto, 1987; Rosenkranz, 1990) the duration of postembryonic development of Africanized bees is shorter than in European bees. Our results show that this difference is not due to the absence of certain stages but rather to the shorter duration of some stages during development. When compared to African bees, *A m adansonii* (Tribe and Fletcher, 1977), and *A m scutellata* (Moritz, 1985), Africanized bees have a slightly longer total duration of pregimaginal stages, a difference of ≈ 18 to 24 h. On this basis, Africanized bees may be considered to occupy a position between European and African bees.

The degree of hybridization in Africanized bees has been determined on the ba-

sis of different parameters such as iso-zyme frequency (Del Lama *et al,* 1988; Lobo *et al,* 1989), morphometry (Buco *et al,* 1987; Daly, 1991), analysis of mito-chondrial DNA (Smith *et al,* 1989; Smith, 1991), and estimates of gene flow among the various European and Africanized pop-ulations (Sheppard and McPheron, 1986; Rinderer *et al,* 1991; Sheppard *et al,* 1991). According to these investigators, the effects of hybridization vary with the in-tensity of gene flow in the different popula-tions; that is, the degree of hybridization is related to the interrelationships among these populations at the limits of their dis-tributions. We would therefore expect a variation in the duration of pre-imaginal de-velopment in Africanized bees related to the degree of hybridization that has oc-curred in the different regions of their geo-graphic distribution. Further studies in re-gions where the different degrees of hybridization have been studied (Sheppard *et al,* 1991) in transition areas will be need-ed to test this hypothesis.

ACKNOWLEDGMENTS

Résumé — Caractérisation des stades de développement préimaginal chez les ouvrières de l'abeille africanisée (*Apis mellifera* L). Le développement préimagi-nal d'*Apis mellifera* a déjà été décrit par plusieurs chercheurs et l'existence de 5 stades larvaires consacrés à l'alimentation et à la croissance est bien établie. Le der-nier stade larvaire peut être subdivisé en diverses périodes, qui impliquent une série de modifications morphologiques et phy-siologiques en liaison avec la métamor-phose. Plusieurs stades ont également été observés pendant la phase nymphale. Di-verses études ont montré que la durée des stades des phases larvaire et nymphale varie d'une race d'abeilles à l'autre. Nous avons étudié en détail ces 2 phases du dé-veloppement préimaginal chez des abeilles africanisées récoltées à Ribeirao Preto, SP, Brésil, et caractérisé les divers stades ainsi que leur durée.

Grâce à un suivi de la ponte, nous avons prélevé des échantillons toutes les 6 h, du 1er stade larvaire jusqu'à l'émer-gence de l'adulte. Les stades larvaires ont tout d'abord été caractérisés par le poids du corps et le diamètre de la tête (fig 1). Ces mesures ont permis de reconnaître 5 groupes, correspondant chacun à un stade larvaire. Les mesures ont porté sur un total de 1 249 ouvrière. Le 5e stade larvaire a été caractérisé par des paramètres supplé-mentaires, tels que la défécation, la posi-tion de la larve dans la cellule et la lon-gueur tibio-tarsale des pattes postérieures. Ces subdivisions ont été classées en 3 pé-riodes d'alimentation (LF1, LF2, LF3) et 3 périodes d'operculation (LS1, LS2, LS3). En LF1, la larve pèse environ 35 mg et à la fin de LF3, qui est le moment où la cellule va être operculée, elle atteint 120 mg. Au stade LS1, la larve est encore enroulée à l'intérieur de la cellule. Elle s'étire progres-sivement et diminue ses mouvements au cours de LS2 et LS3. La défécation a lieu durant ces stades. En LS1, l'intestin est complètement rempli, la défécation propre-ment dite commence en LS2 et se poursuit en LS3. La longueur tibio-tarsale est de 0,8 à 1 mm en LS1 et reste inchangée jusqu'à la fin de LS3. La période pré-nymphale (stades PP) commence lorsque l'intestin est complètement vide, la nymphe immobile et que l'apolyse commence dans la région céphalique. La longueur tibio-tarsale passe à 1,20–1,44 mm (PP1). En PP2, elle augmente jusqu'à 1,80–2,00 mm

et reste inchangée en PP3. L'apolyse progresse et culmine au moment de la mue nymphale à la fin de PP3.

Des échantillons de nymphes ont également été prélevés toutes les 6 h à partir d'une ponte contrôlée et 7 stades (tableau III) ont été caractérisés d'après la couleur de l'œil et celle du thorax.

Par cette caractérisation, on a pu ainsi déterminer la durée des stades (fig 3), avec une précision de 6 h. Si l'on compare les stades décrits ici avec ceux décrits pour les abeilles européennes, on trouve peu de différences dans la durée des 4 premiers stades larvaires. Au 5e stade pourtant, on s'aperçoit que la période LF3 (operculation de la cellule) est plus courte d'environ 6 h chez l'abeille africanisée. Le même phénomène se produit pour les prénymphes. D'autres différences sont visibles aux stades Pw et Pb pendant la phase nymphale, chaque stade étant plus court de 6 h par rapport à l'abeille européenne *(A m carnica)*. La différence totale atteint environ 24 h, et cela n'est pas dû à l'absence de stades, mais plutôt à la durée réduite de certains d'entre eux. Comparée aux abeilles africaines, la durée du développement pré-imaginal des abeilles africanisées est plus longue d'environ 24 h. De ce point de vue, les abeilles africanisées semblent donc occuper une position intermédiaire entre les européennes et les africaines.

abeille africanisée / développement post-embryonnaire / durée de développement / stade larvaire

Zusammenfassung — Charakterisierung der präimaginalen Entwicklungsstadien bei Arbeiterinnen der afrikanisierten Honigbiene (*Apis mellifera* L). Die präimaginale Entwicklung von *Apis mellifera* wurde schon von mehreren Forschern beschrieben. Es wurden fünf larvale Häutungsstadien festgestellt, mit den vorwiegenden Aktivitäten des Fressens und des Wachstums. Das letzte larvale Stadium kann in verschiedene Perioden unterteilt werden, die mit einer Serie von morphologischen und physiologischen Veränderungen im Zuge der Metamorphose zusammenhängen. Auch in der Puppenphase können mehrere Stadien beobachtet werden. Einige Studien haben Unterschiede in der Länge der Larven- und Puppenstadien bei verschiedenen Bienenrassen gezeigt. Wir haben diese beiden Phasen der präimaginalen Entwicklung bei afrikanisierten Bienen aus Ribeirao Preto (SP, Brasilien) untersucht und die verschiedenen Stadien sowie deren Dauer beschrieben.

Die Proben wurden unter kontrollierter Eiablage in 6-stündigen Intervallen vom ersten Larvenstadium bis zum Schlüpfen der Biene gesammelt. Die Larvenstadien wurden zunächst nach ihrem Körpergewicht und dem Kopfdurchmesser (Abb 1) charakterisiert, wobei fünf Gruppen entsprechend jeder Häutung festgestellt werden konnten. Insgesamt wurden 1249 Arbeiterinnen gemessen. Das 5.Stadium wurde nach zusätzlichen Merkmalen, wie Defäkation, Lage der Larve in der Zelle und Tibio-Tarsallänge des Hinterbeins unterteilt. Diese Unterteilungen wurden als die folgenden Perioden klassifiziert: Freßperiode 1, 2 und 3 (LF1, LF2, LF3). Während der LF1 wiegt die Larve ungefähr 35 mg; am Ende von LF3, zur Zeit der Zellverdeckung, erreicht sie 120 mg. LS1, LS2 und LS3 entsprechen den Perioden ab Beginn der Zellverdeckung. Während LS1 liegt die Larve noch eingerollt in der Zelle. Während der Perioden LS2 und LS3 streckt sie sich in der Zelle allmählich aus und sie verringert ihre Bewegungen. Während dieser Perioden erfolgt die Defäkation. Während LS1 ist der Darm noch vollständig gefüllt, die eigentliche Defäkation beginnt während LS2 und schreitet wäh-

rend LS3 fort. Die Tibio-Tarsallänge beträgt während LS1 0.8-1.0 mm und bleibt bis zum Ende von LS3 unverändert. Die Vorpuppenperiode beginnt, sobald der Darm vollständig leer ist; die Puppe liegt völlig bewegungslos, und die Apolyse beginnt in der Kopfregion. Die Tibio-Tarsallänge vergrößert sich auf 1.20-1.44 mm (PP1). Während der PP2 wächst die Tibio-Tarsallänge auf 1.80-2.00 mm, aber während PP3 bleibt sie unverändert. Die Apolyse schreitet fort und erreicht in der Puppenhäutung am Ende von PP3 ihren Höhepunkt.

An den in 6-stündigen Intervallen ab der kontrollierten Eiablage gesammelten Proben konnten nach der Farbe von Augen und Thorax 7 Puppenstadien unterschieden werden (Tabelle III).

Auf der Grundlage dieser Klassifizierung konnte die Dauer der verschiedenen Stadien mit einer Genauigkeit von 6 Stunden bestimmt werden (Abb 3). Bei dem Vergleich mit den Angaben über europäische Rassen bestanden für die ersten vier Larvenstadien nur geringe Unterschiede. Für das 5.Stadium ergab sich jedoch bei der Periode LF3 (Zellverdeckelung) für unser Material eine Verkürzung von 6 Stunden, und dasselbe war bei den Vorpuppen festzustellen. Unterschiede waren auch während der Puppenphase bei den Perioden Pw und Pb zu beobachten, die jede um 6 Stunden kürzer waren als bei europäischen Rassen *(A m carnica)*. Es ergab sich ein Gesamtunterschied von 24 Stunden, der aber nicht durch das Fehlen von Häutungsstadien entstand, sondern vielmehr durch die Verkürzung jedes einzelnen Stadiums bei den afrikanisierten Bienen. Bei einem Vergleich mit originalen afrikanischen Bienen war die präimaginale Entwicklung der afrikanisierten Bienen etwa um 24 Stunden länger. Es scheinen also die afrikanisierten Bienen in dieser Hinsicht eine Mittelstellung zwischen euro-päischen und afrikanischen Bienen einzunehmen.

Afrikanisierte Biene / Präimaginale Entwicklung / Entwicklungsdauer / Larvale Stadien

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