EVOLUTION & DEVELOPMENT 11:3, 269–277 (2009)
DOI: 10.1111/j.1525-142X.2009.00329.x

Endocrine signatures underlying plasticity in postembryonic development of a lower termite, *Cryptotermes secundus* (Kalotermitidae)

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SUMMARY Wood-dwelling termites are characterized by an extremely high and unique developmental flexibility that allows workers, which are immatures, to explore all caste options. The endocrine signatures underlying this flexibility are only vaguely understood. We determined juvenile hormone (JH) and ecdysteroid hemolymph titers during postembryonic development and in terminal instars of the drywood termite *Cryptotermes secundus* using field and laboratory colonies. Postembryonic development is characterized by a drop in JH titers at the transition from larval (individuals without wing buds) to nymphal (individuals with wing buds) instars. JH titers were low in winged sexuals and reproducing primary reproductives (<200 pg/μl) but were by an order of magnitude higher in neotenic replacement reproductives. The unique regressive molts of termites seem to be characterized by elevated JH titers, compared with progressive or stationary molts. Ecdysteroid titers were generally low in nymphal instars and in primary reproductives (<50 pg/μl). It was only during the third and fourth nymphal instars and in winged sexuals where some individuals showed elevated ecdysteroid titers. These results are the most comprehensive endocrinological data set available for any lower termite, with the potential to serve as baseline for understanding the extreme developmental flexibility underlying the evolution of social life in termites.

INTRODUCTION

In terms of their phenotypic plasticity, termites are probably only matched by the ants, which have reached a similar pinnacle of social evolution in a completely distinct clade. And like in ants, this morphological variation among the different castes of a termite colony is primarily triggered by environmental stimuli, so that reproductives, workers, and soldiers develop within a colony essentially on the same genetic background. While there are only a few termite species for which a genetic basis to caste determination has been proposed (Hayashi et al. 2007), in the majority of genera the divergence in developmental pathways is triggered by environmental stimuli, such as social interactions, food availability, or season (Roisin 2000; Korb and Hartfelder 2008).

Recent taxonomic revisions firmly nest the termites within the cockroaches (Blattodea), more specifically as a sister taxon to the wood-dwelling cockroaches Cryptocercidae (Klaas and Meier 2006; Inward et al. 2007). The extant termites seem to have exploited the developmental plasticity already incipient in these cockroaches, which can respond to deteriorating food conditions by undergoing supernumerary molts (Bell et al. 2007). Consequently, to understand the evolutionary background of the developmental plasticity seen in termites, an obvious group to study are the wood-dwelling termites. These termites exhibit the ancestral life type of spending the entire colony life in a single piece of wood that serves both as food source and shelter (one-piece nesters *sensu* Abe 1987).

These wood-dwelling termites show fantastic developmental plasticity, even more so than the higher termites (Termitidae), in which developmental pathways have become more fixed leaving fewer options to an immature after the early larval stages (Noirot 1990; Roisin 2000). In the wood-dwelling drywood termites, all workers are ontogenetically totipotent immature, hereafter called "false workers" (Korb and Hartfelder 2008) that can develop (i) via two molts into sterile soldiers, (ii) via several molts into winged dispersing sexuals (alates) that found a new colony, (iii) via a single molt into neotenic replacement reproductives that inherit the natal breeding position when the same sex reproductive of the colony dies, or (iv) there is no change and they continue to be a false worker after the following molt(s) (Fig. 1). This flexibility in development is brought about through three molting types: (i) progressive molts which characterize the gradual

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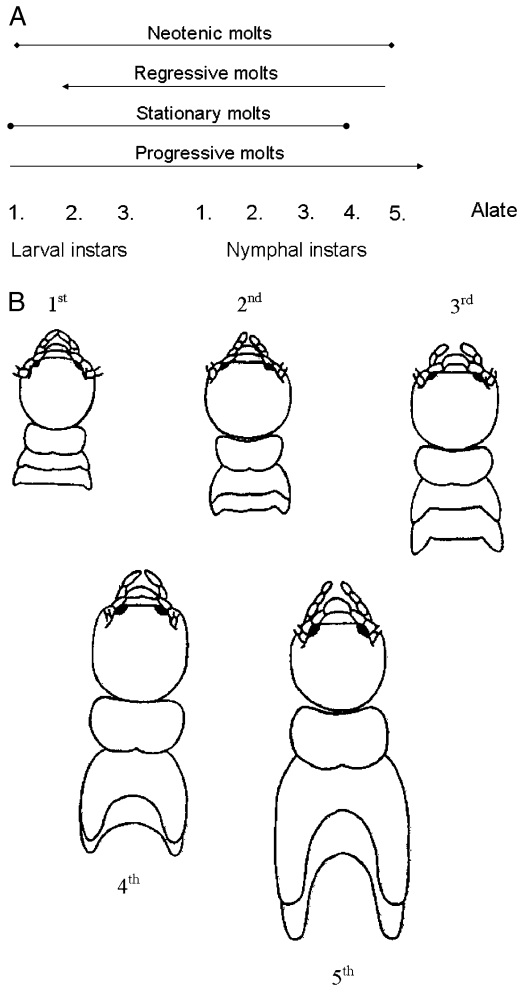

Fig. 1. (A) Developmental trajectories of *Cryptotermes secundus*. The diagram shows false worker stages consisting of three larval (without wing buds) and five nymphal (with wing buds) instars, and their ability to molt progressively, stationary, regressively, or into neotenic replacement reproductives (order of lines: from bottom to top). All these instars have the ability to molt progressively into the next instar or into a neotenic reproductive. In addition first instar larvae up to fifth instar nymphs can undergo stationary molts, and second instar larvae up to the fifth nymphal instar can molt regressively. (B) Classification of nymphal instars (first–fifth) according to wing bud development and shape (modified after Sewell and Watson 1981).

development from the first postembryonic stage via several instars into an adult; they are associated with an increase in body size and morphological development; (ii) stationary molts which are intermittent molts that are associated with a lack of increase in body size and morphological development; and (iii) regressive molts that are characterized by a decrease in body size and/or regression of morphological development, generally a reduction in wing bud size in nymphal instars. While progressive molts are the rule in insects and stationary molts are not uncommon, the option to pass through a regressive molt is clearly unique to lower termites. This plasticity in developmental options and molting types makes wood-dwelling termites not only a fascinating breed, but also a major challenge to any model on endocrine regulation in insect development (Nijhout 1994; Truman and Riddiford 2002).

Notwithstanding, endocrinological studies on termite development in general are relatively scarce, with application experiments prevailing on applied aspects, that is ways to eradicate termites by offsetting caste ratios through treatment with juvenile hormone (JH) and JH analogs (Hrdy 1976; Hrdy et al. 2006). Such experiments early on showed the potential of JH to induce molts leading to the soldier phenotype (Hrdy 1976; Hartfelder and Emlen 2005), and it is this property that has been used to reveal cellular and molecular underpinnings of soldier development in a variety of termite species belonging to different families and genera (reviewed by Miura et al. 1999; Miura 2001; Hartfelder and Emlen 2005; Zhou et al. 2006a; Korb and Hartfelder 2008).

As mentioned above, these studies relied on applications of JH or JH analogs, and despite having generated important insights into molecular mechanisms underlying soldier development (Miura et al. 1999; Scharf et al. 2005; Zhou et al. 2006a, b, 2007) they require validation from data on actual hormone titers. This requirement has been incisively argued by Zera (2006, 2007), who showed the importance of actual JH titer measurements in microevolutionary studies on wing polyphenism in gryllids. In termites, actual hormone titer determinations were, with two notable exceptions (Lanzrein et al. 1985a; Okot-Kotber et al. 1993), initiated only relatively recently, and most of these studies had their focus either on soldier development (Park and Raina 2004, 2005; Mao et al. 2005; Elliott and Stay 2007), or on reproductive physiology (Brent et al. 2005; Brent 2007; Elliott and Stay 2007, 2008).

This study had two principal aims: first, we wanted to describe the time course of the JH and ecdysteroid titers throughout postembryonic development (starting from third instar larvae) for the different castes of the drywood termite *Cryptotermes secundus* (Kalotermitidae); second, we wanted to see whether we could find endocrine correlates for the different molting types (progressive, stationary, or regressive) from posterior probabilities of molting events observed in colonies during different seasons. For *C. secundus* we know for instance that the frequencies of molting types for each instar differ predictably during the course of the year (Korb and Katrantzis 2004). A third, related aim was to analyze whether individuals sampled from field and laboratory colonies differed in their JH titers. Laboratory colonies are important tools for manipulation experiments. Yet, for drawing evolutionary conclusions it is important that laboratory data are backed up by actual field data.

MATERIALS AND METHODS

Collection of termites in the field—field experiments

In July 2007 we collected dead mangrove trees (*Ceropis tagal*) that could potentially harbor *C. secundus* colonies near Palmerston-Channel Island in Darwin Harbor (Northern Territory, Australia; 12°30′S 131°00′E) (for more details, see Korb and Katrantzis 2004). They were carefully split with hammer and chisel to obtain complete *C. secundus* colonies. After the caste composition was determined, individuals were classified into instars according to size and wing bud development (Korb and Katrantzis 2004). In total, three larval worker (first–third larval instar; in termites, individuals without wing buds) and five nymphal instars (first–fifth nymphal instar; in termites, individuals with wing buds) exist in *C. secundus* before the imaginal molt into an alate (Korb and Katrantzis 2004) (Fig. 1). During the collection period before the annual nuptial flight all instars were present (Korb and Katrantzis 2004).

Establishment of laboratory colonies—laboratory experiments

Colonies used for hormone titer measurements had been collected during previous years at the same site where the field experiments were carried out (Korb and Lenz 2004). These colonies had been transferred to Germany and kept in a climate chamber at 28°C and 70% relative humidity with a 12 day/night cycle (for more details see Korb and Katrantzis 2004; Korb and Schmidinger 2004). These conditions are appropriate for *Cryptotermes* and do not affect growth or development of colonies. As in the field, laboratory colonies were extracted from their wood, colony composition was determined, and individuals were classified into instars, before they were punctured for hemolymph sampling. Because there was no further experimental manipulation, the termites collected from laboratory colonies were considered to represent near-natural conditions.

Collection of hemolymph

From the field and laboratory colonies, individuals from all instars were taken according to availability. Termites were immobilized on a wax-preparation-plate by pressure from crosswise inserted entomological pins. The abdomen was punctured with a thin entomological pin between tergites and protruding drops of hemolymph were absorbed into calibrated glass capillaries (5 µl single-use capillary pipettes; neoLab, Heidelberg, Germany). Because a volume of at least 2 µl hemolymph was required for hormone titer measurements, one to four individuals had to be pooled, depending on the instar. For the fourth (penultimate) and fifth (ultimate) nymphal instar and the terminal stages, single individuals provided sufficient hemolymph for the assays. First and second larval instars were too rare and/or too small, and thus were not sampled. For JH measurements, hemolymph aliquots of 1–5 µl were immediately dissolved in 0.5 ml acetonitrile, whereas for ecdysteroid titer determination, aliquots of 2 µl were added to 200 µl methanol. These samples were stored at − 20 °C in teflon stoppered screw cap glass vials.

JH titer analysis by radioimmunoassay (RIA)

JH was extracted following a liquid-phase separation protocol (Huang et al. 1994). Briefly, 1 ml NaCl (0.9%) and 1 ml hexane were added to the acetonitrile extract. After vigorous vortexing, the phases were separated by centrifugation (700 *g*). The hexane phase was collected and the extraction was repeated twice. The pooled hexane phases were dried by vacuum centrifugation and the residues were redissolved in 100 µl toluene and transferred to RIA glass vials. Before starting the RIA, the solvent was removed by vacuum centrifugation.

The JH-specific antiserum was diluted 1:1250 in phosphate buffer supplemented with bovine serum albumin (0.1%) and rabbit immunoglobulin G (0.1%). The assays were performed with [10-3H(N)]-JH III (spec. activity 19.4 Ci/mmol, Perkin Elmer Life Sciences, Waltham, MA, USA), diluted in phosphate buffer to 6000–6500 cpm/50 µl. JH III (Fluka, Munich, Germany) was used as nonradioactive ligand. Standard curves were set up to cover a 50 pg–10 ng range. The RIA procedure followed the protocol established by Goodman et al. (1990). Samples were incubated overnight at 4°C before separation of antibody-bound JH from free JH by addition of ammonium sulfate (50% final concentration). Standard curve values were log/logit transformed so that JH titers of the samples could be calculated by linear regression and be expressed as JH-III equivalents (pg/µl hemolymph). JH-III is the only JH moiety released by termite corpora allata (Brent et al. 2005; Yagi et al. 2005) and detected in hemolymph (Park and Raina 2004; Brent et al. 2005; Cornette et al. 2008).

In total, we analyzed 153 JH samples, half from the field (from a total of 26 colonies) and half from the laboratory (from a total of 21 colonies). The samples comprised third instar larvae up to fifth instar nymphs, different types of reproductives (newly molted and sclerotized, older alates, primary reproductives, and neotenics) and a soldier sample. We did not find presoldiers in the field or laboratory colonies, so we could not include such a sample.

Ecdysteroid titer analysis by radioimmunoassay

The methanol samples were centrifuged (14,000 *g*, 4°C, 10 min) to pellet precipitated hemolymph proteins before the supernatants were transferred to 1-ml glass vials. After solvent evaporation by vacuum centrifugation, the ecdysteroids were quantified by RIA, as described previously (Feldlaufer and Hartfelder 1997; Pinto et al. 2002), using an antiserum prepared against a hemisuccinate derivative of ecdysone (Bollenbacher et al. 1983; Warren and Gilbert 1986). The antiserum was diluted to a final concentration of 0.075% in borate buffer (pH 7.4) containing 10% rabbit serum. The radioactive ligand was [23,24-3H(N)]ecdysone (spec. activity 102 Ci/mmol, NEN). Samples were incubated overnight at 4°C before precipitation of antibody-bound ecdysteroids by addition of saturated-ammonium sulfate. Standard curves were established using 20-hydroxyecdysone (20E, Simes, Milan, Italy) as nonradioactive ligand, covering a range from 25 pg to 2 ng 20E. Results are, therefore, expressed as 20E equivalents (pg/µl hemolymph), which was identified as the predominant RIA active ecdysteroid moiety in the termite *Reticulitermes flavipes* (Okot-Kotber et al. 1993). A total of 38 samples, postembryonic stages and adults collected from four field colonies, were submitted to the ecdysteroid RIA analyses.

Determination of molting type frequencies in field and laboratory colonies

Because of the seasonal development of alates, the frequency of the different molting types (progressive, stationary, regressive) differs predictably between instars and seasons. For *C. secundus* the frequencies of molting types per instar and season were previously determined (Korb and Katrantzis 2004), and we used these values for our field season experiment in July 2007.

As the period of our laboratory experiments was only partially covered by the cited study, we determined the frequencies of molting types for the laboratory experiment directly in our experimental colonies. Individuals that were close to molting—indicated by their whitish opaque appearance—were classified into instars (Fig. 1). They were then separated into 2.0-ml vials and kept with a piece of humid wood as a supply for food and water until they molted. After the molt and before reintroduction into their natal colony, molted individuals were reclassified into instars and their molting type scored as either progressive, stationary, or regressive. This procedure does not affect the development of individuals (Korb and Katrantzis 2004).

Statistical analyses

All statistical analyses were performed using SPSS 15.0 or GraphPad Prism and all tests were two-tailed. When data did not meet requirements for parametric testing, nonparametric analyses were performed. Otherwise, results from parametric analyses are given. For all data, qualitatively the same results were obtained when testing parametrically or non-parametrically. *P*-values were corrected for multiple comparisons using the step-up false discovery rate (FDR) approach, a method suggested to overcome problems of Bonferroni's correction procedures (Benjamini and Hochberg 1995; Garcia 2004).

RESULTS

Morphogenetic hormone titers in postembryonic stages of *Cryptotermes secundus*

JH titers were determined by RIA for third instar larvae and for the subsequent five nymphal instars. The fifth is the ultimate instar before the molt to the winged adult. Titer data from a total of 127 samples (field and laboratory) are shown in Fig. 2. The upper panel (Fig. 2A) (representing the mean values and standard errors for the six developmental stages) shows that the hemolymph JH titer significantly decreased at the transition from the larval to the nymphal instars (ANOVA: $F_{5\,126} = 8.88$, after step-up FDR: $P < 0.05$, Tukey's *post hoc* test for third larval against all nymphal stages: $P < 0.01$). This drop in JH titers is most marked in the second nymphal instars, but thereafter, some outliers are within the range of the larval samples (Fig. 2B).

Ecdysteroid titer measurements covering the five nymphal instars were obtained from a total of 28 hemolymph samples from individuals collected from field colonies (Fig. 3). All but four of the samples had titers below 100 pg 20E equivalents/µl hemolymph. This was also reflected in the statistical analysis (Kruskal–Wallis test: $\chi^2_4 = 4.99$, $P = 0.288$), which did not indicate significant differences between the nymphal instars.

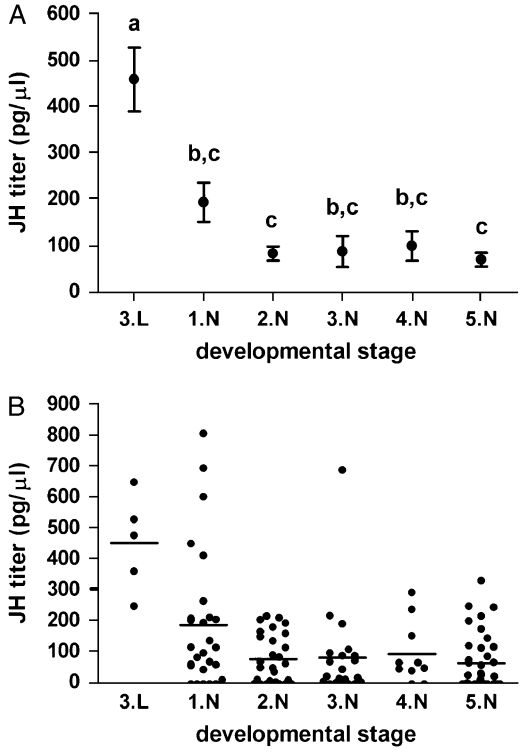

Fig. 2. Juvenile hormone titers in postembryonic stages of *Cryptotermes secundus*, from the third (last) larval (3L) to the fifth (last) nymphal instar from field and laboratory colonies. Data are represented as (A) mean and standard errors for each instar, different letters indicating significant differences (ANOVA, $P < 0.05$), and (B) as plots of individual titer measurements; means are the same as in A and are shown here as horizontal bars.

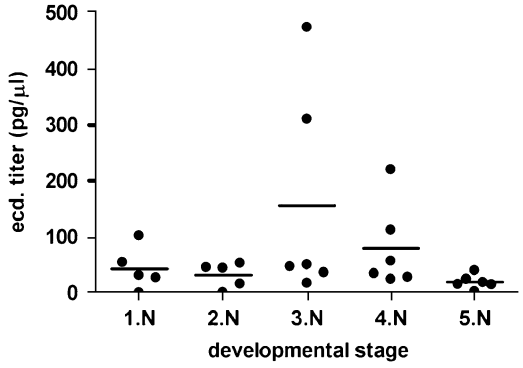

Fig. 3. Ecdysteroid titers in the nymphal instars of *Cryptotermes secundus* from field colonies. The data are plotted as individual titer measurements, means are represented by horizontal bars.

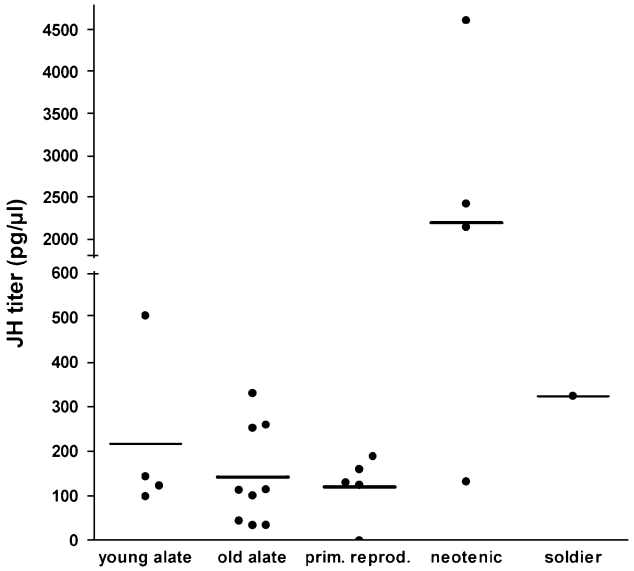

Fig. 4. Juvenile hormone titers in terminal castes of *Cryptotermes secundus* from field and laboratory colonies. Alates were collected from the natal nest before the nuptial flight and were separated into young (directly after the molt) and old (sclerotized) alates. Primary reproductives are derived from alates that founded a new colony. Neotenic reproductives are derived from nymphs that did not develop their wing buds, they replaced the primary reproductives in the natal colony. Data are plotted as individual titer measurements to show the high variation in titer levels. The axis was interrupted at 600 pg/µl so that the extremely high titer levels of neotenics could be shown. Means are represented as horizontal bars.

JH and ecdysteroid titers in terminal castes of *Cryptotermes secundus*

We determined JH titers for a total of 26 samples (19 from field and six from laboratory colonies), comprising young (newly molted) and older (sclerotized) alates, primary reproductives, neotenic reproductives, and one sample for the soldier caste. JH titers did not differ significantly between alates and primary reproductives (Mann–Whitney *U*-test: $N_{\text{all alates}} = 10$, $N_{\text{primaries}} = 5$, $U = 22$, $P = 0.713$; Fig. 4). This was unexpected, considering that the alates are still in the parental nest, before their nuptial flights, whereas primary reproductives had shed their wings after successfully mating, established their own nest and were actively reproducing. These primary reproductives were at least 1-year-old and their relatively low JH titers stood in stark contrast to those of the other reproductive morph, the neotenic reproductives, where three out of four individuals had extraordinarily high JH titers, above 2 ng/µl hemolymph. Accordingly, neotenic reproductives had significantly higher JH titers than primary reproductives (Mann–Whitney *U*-test: $N_{\text{primaries}} = 5$, $N_{\text{neotenics}} = 4$; $U = 0.0$, after step-up FDR: $P < 0.05$). After the imaginal molt, the JH titers seemed to drop from young alate, via old alate to reproducing primary but no significant correlation was found (Spearman's rank correlation: $N = 15$, $P = 0.721$). The JH level for the soldier sample was within the range of that for primary reproductives, but in this case sample size was too small for meaningful comparisons.

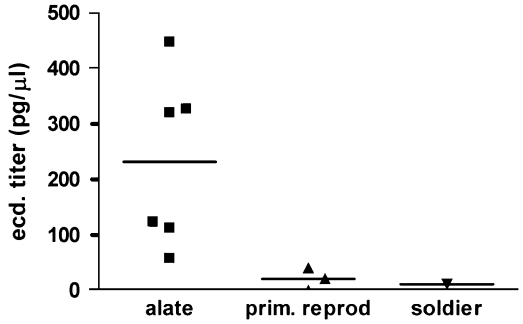

Fig. 5. Ecdysteroid titers in terminal castes of *Cryptotermes secundus* from field colonies. Data are plotted as individual titer measurements, showing a bimodal distribution for the alates. Means are represented as horizontal bars.

Ecdysteroid titers were measured for alates (six samples) and primary reproductives (three samples) and a single soldier sample (Fig. 5), revealing an apparent dichotomy in the alates, with half of the hemolymph samples >300 pg/µl and the other <150 pg/µl. Primary reproductives and the soldier sample had only very low ecdysteroid titers.

Because the molt from the last nymphal instar to the alate is associated with major morphological change we, furthermore, compared the two groups directly with respect to their hormone titers. After the imaginal molt the JH titer increased compared with the preceding nymphal stage (Mann–Whitney *U*-test: $N_{\text{5th nymph}} = 36$, $N_{\text{all alates}} = 13$; $U = 106$, after step-up FDR: $P < 0.05$). The comparison for the ecdysteroid titers showed a similar result, revealing a significant increase in ecdysteroid titers after the adult molt (Mann–Whitney *U*-test: $N_{\text{nymphal}} = 6$, $N_{\text{alates}} = 6$; $U = 0.0$, after step-up FDR: $P < 0.05$).

Comparison of JH titers between field and laboratory colonies: the nymphal stages

We compared the JH titer developmental profiles for the five nymphal stages collected from laboratory colonies with those from field colonies. Nymphs from the laboratory and the field colonies showed overall very similar JH titer profiles (Fig. 6). Nevertheless, a differentiated comparison was necessary to account for the different molting type frequencies between the field and laboratory colonies caused by different sampling seasons (Table 1). For the fifth nymphal instar (ultimate nymph), which always molts with a probability of $>$ 80% progressively into an alate, the JH titer values did not differ significantly between the field and the laboratory (*t*-test for independent samples: $N_{\text{lab}} = 17$, $N_{\text{field}} = 19$, $t_{1,34} = 1.52$, $P = 0.137$). Because the other instars differed to various degrees in their molting type frequencies, we used this information to ask whether we find specific endocrine signatures associated with molting types.

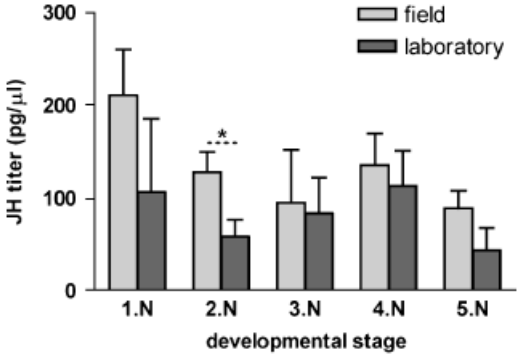

Fig. 6. Juvenile hormone titers in the nymphal instars of *Cryptotermes secundus*, separated according to their sampling from field or laboratory colonies. For the fifth instar nymphs molting type frequencies were similar for the field and laboratory season and we also did not find differences in JH titers. For the second instar nymphs molting type frequencies differed significantly ($^*P<0.05$) between the laboratory and field samples due to the different sampling seasons (see Table 1).

Putative endocrine signatures for regressive molts

The molting type data (Table 1) showed that it was only for the second nymphal instar that molting type frequencies differed considerably and consistently between the laboratory and field samples due to the different sampling seasons. During the field season >40% of the second instar nymphs molted regressively, with <10% molting progressively. In contrast, during the laboratory season >40% of the nymphs molted progressively and only 17% molting regressively. The JH titers were significantly higher in second instar nymphs collected from the field colonies (*t*-test for independent samples: $N_{lab} = 19$, $N_{field} = 9$; $t_{1,26} = 2.30$, after step-up FDR: $P<0.05$; Fig. 6). This indicates that regressive molts are associated with relatively higher JH titers than progressive or stationary molts. Interestingly, the ecdysteroid titer levels in this instar, which had the highest frequency of regressive molts across all instars (Table 1), were among the lowest found for the entire set of nymphal instars collected in field colonies (see Fig. 3).

Table 1. Frequency of molting types [in %] from the observed instar to the next, for the five nymphal instars of *Cryptotermes secundus*

	Field				Laboratory			
Instar	Pro	Stat	Reg	*N*	Pro	Stat	Reg	*N*
1st nymphal instar	(33.3)	(66.6)	—	3	26.7	67.8	5.4	202
2nd nymphal instar	7.1	50.0	42.9	14	43.6	38.7	17.8	163
3rd nymphal instar	36.6	33.3	30.1	23	27.4	47.2	25.4	252
4th nymphal instar	48.2	30.8	21.0	25	81.7	4.3	14.0	93
5th nymphal instar	98.0	2.0	0	26	84.9	3.5	11.6	172

The frequencies were determined for laboratory colonies, the data for the field season are taken from Korb and Katrantzis (2004).
Pro: progressive molt, Stat: stationary molt, Reg: regressive molt, *N*: sample size. Data in brackets were derived from a small sample size.

DISCUSSION

The wood-dwelling termites represent the vantage point for the ecological and evolutionary success of this clade of social insects, and this success is directly associated with their amazing developmental plasticity. Yet, except for soldier development, the endocrine mechanisms underlying such plasticity are little understood. As a first step to address this question we checked whether hormone titers in laboratory colonies of *C. secundus* might differ from termites sampled under field conditions. For this purpose we made use of data collected previously on molting type frequencies in field colonies (Korb and Katrantzis 2004) and we compared these molting type frequencies to those observed in laboratory colonies. The general development of individuals was not affected by the laboratory conditions (Korb and Katrantzis 2004) and, correspondingly, we also did not note major differences in JH hemolymph titers between field and laboratory colonies for most of the nymphal instars, except for the second instar, where field colonies had higher mean JH titers than laboratory colonies. Because molting type frequencies differed exactly for this instar, we consider that the higher JH titers in the field samples could be related to the higher frequency of regressively molting individuals. By contrast, for the fifth nymphal instar where molting type frequencies did not differ between the field and laboratory season, JH titers did not differ between field and laboratory samples. So, when accounting for these season-related differences in molting types we can conclude that termites sampled from unmanipulated laboratory colonies are valid proxies for field samples.

Endocrine system activity in postembryonic stages

Having confirmed this, we could next investigate the time course of the JH and ecdysteroid titers during postembryonic development, specifically from the third (last) larval instar to the fifth (last) nymphal stage. Instars without wing buds (larval instars) were characterized by a higher JH titer than instars with wing buds (nymphal instars). Similarly, Cornette et al. (2008) had recorded a decline in JH titers from the larval to the nymphal instars for *Hodotermopsis sjostedti*, and there was an increase as they passed the imaginal molt, remembering that this termite has only a single nymphal instar before the imaginal molt.

Probably the most challenging question with respect to termite developmental plasticity is their capacity to undergo not only progressive or stationary, but also regressive molts,

the latter being a molting type restricted to lower termites. Furthermore, the progressive molt may lead into different developmental pathways: to alates, neotenics, or soldiers. Tackling the endocrine underpinnings of molting type variation is not trivial because it requires that one can predict the trajectory that a termite follows at the next molt. So far, this is only clear for the presoldier-to-soldier developmental pathway, reviewed by Miura (2004), but not for the other developmental trajectories. In this study we made use of molting type frequency estimates for termites collected during the field versus the laboratory season. The second nymphal instar showed marked differences in molting type frequencies, and setting this in conjunction with the respective JH titer measurements we conclude that the higher JH titers observed in the field samples may be associated with a higher frequency of regressive molts at this developmental stage, given certain environmental conditions. Even though this is a small dataset, it is the first one to support not only the hypothesis that regressive molts are associated with elevated JH titers, but also the unproven idea that high JH titers during nymphal stages prevent the development of termite "workers" into sexuals (Lüscher 1956, 1974; Springhetti 1969; Stuart 1979). It is, however, important to add a *caveat* here. Our data, as well as those of most other studies, represent mean values of hormone titers for developmental stages that can last several weeks. As recently shown for the termite *Reticulitermes flavipes*, rates of JH synthesis can vary considerably during developmental stages leading up to the differentiation of soldiers or apterous neotenics (Elliott and Stay 2008).

The high JH titers found in third instar larvae and the subsequent drop in hormone levels as they passed through the nymphal instars is interesting. In this aspect this basal termite differs critically from cockroaches, the clade within which termites are now taxonomically situated (Inward et al. 2007). In the ovoviviparous cockroach *Nauphoeta cinerea,* very high JH titers were detected in embryos that had undergone dorsal closure. But thereafter the titer sharply drops as they complete embryonic development and become first instar nymphs, and continues at low levels during the remainder of the nymphal stages (Lanzrein et al. 1984, 1985b). In this and other cockroaches, the JH titer only increases again after the imaginal molt. Thus, the JH titer pattern observed during postembryonic larval development in *C. secundus* and *H. sjostedti* (Cornette et al. 2008) resembles that seen in cockroaches during the end of embryonic development.

This difference in developmental endocrinology between termites and cockroaches suggests that during their early evolution from wood-nesting cockroaches, termites may have modified their developmental chronology and, like the holometabolan clades (Truman and Riddiford 1999, 2002), may have de-embryonized a pronymphal stage and prolonged it into larval stages. The pronymphal stage, present in some of the more primitive hemimetabolans, is a short development stage between the conclusion of embryonic development and eclosion of the first nymphal instar from the egg. It is a cornerstone of the Berlese-Jeshikov-hypothesis on the origin of the holometabolan mode of development (for discussion see Novák 1975; Truman and Riddiford 1999, 2002). Obviously, this is, at present, a bold suggestion, but the biology of the early larval instars of termites, which are frequently referred to as dependent larvae (reviewed in Noirot 1990; Shellman-Reeve 1997; Korb and Hartfelder 2008), as well as the lack of a description of a pronymphal stage in termite embryos (for *Kalotermes flavicollis* and *Zootermopsis nevadensis* see Striebel 1960); for *Cryptotermes brevis* see Kawanishi 1975) may strengthen this conclusion.

Endocrine system activity after the terminal molt

After the imaginal molt, the JH titers seemed to drop from the young alate, via old alate to the primary reproductive stage, but no significant correlation was found (Fig. 4). In contrast, neotenics of *C. secundus* appeared to be literally filled with JH. The JH titers of soldiers were within the range of that for primary reproductives. This is in accordance with results for the subterranean rhinotermitid *Coptotermes formosanus* in which JH titers are only increased in presoldiers, but did not differ between workers and soldiers (Park and Raina 2004). The results for *C. secundus* neotenics contrast with those reported for *H. sjostedti*, where neotenics had lower JH titers than larvae and nymphs (Cornette et al. 2008). However, they are in accordance with JH synthesis rates in *R. flavipes* where neotenics far exceeded those of workers, soldiers, and even presoldiers (Elliott and Stay 2008). Thus these three termite species show major differences in JH titers between neotenics and the other castes (despite opposite directionalities). The apparently opposite constellation (*Reticulitermes*/*Cryptotermes versus Hodotermopsis*) is surprising and is not reflected in phylogenetic position or life type.

Our study is the first one to directly compare primary and neotenic reproductives and, interestingly, elevated JH titers were exclusively found in neotenic but not in primary reproductives, even though these two reproductive types do not differ in their fecundities (Korb and Schneider 2007). Further questions that still need to be resolved for the reproductive types of *C. secundus* are whether there are differences in JH titers between the sexes, as shown for *H. sjostedti* (Cornette et al. 2008), and what may be the source and role of the elevated ecdysteroid titers in the alates of *C. secundus*, contrasting with the low titers in primary reproductives.

The role of ecdysteroids in insect reproduction has mainly been studied in dipterans, where ecdysteroids promote various aspects of follicle development, especially vitellogenesis (Raikhel et al. 2005). In hemimetabolans, the role of ecdysteroids in female reproductive physiology is less clear, and most of the ecdysteroids produced by the follicle epithelium

cells may actually become deposited in the growing eggs, serving as stocks for the regulation of embryonic molts (Hoffmann and Lageux 1985). In cockroaches, where JH is the major regulator of female reproductive physiology (reviewed in Engelmann 1986; Raikhel et al. 2005) ecdysteroids may be playing a dual role, (i) in oogenesis, where they may inhibit vitellogenesis (Engelmann 2002), but promote chorion formation (Zhu et al. 1983), and (ii) in embryogenesis, where they may affect dorsal closure (Lanzrein et al. 1984, 1985b), concomitant with the deposition of larval cuticle. Considering these diverse roles played by ecdysteroids in female cockroaches, the possible function of the elevated ecdysteroid titers observed in *C. secundus* alates, as compared with primary reproductives, is hard to predict at present, requiring further investigation. In general, interpreting hormonal changes in young adults is a complicated matter because of the overlap of maturation processes after the imaginal molt with decisions on reproductive tactics (Dingle and Winchell 1997).

General conclusions

By RIA measurements of JH and ecdysteroid titers we obtained a major dataset covering postembryonic and adult/terminal stages of the termite *C. secundus*. We consider these results to be important for two reasons: first, they are the largest database on hormone titers in a termite with a phylogenetically ancestral life type, associated with highly flexible development; second, the samples were obtained from unmanipulated colonies in the field and in the laboratory. This made it possible to validate sampling procedures from laboratory colonies and to gain insights on endocrine signatures underlying the different developmental options that larvae and nymphs have. For second instar nymphs we could show that higher JH titers were associated with a higher frequency of regressive molts, lending support to previous hypotheses postulating that elevated JH titers during critical periods may prevent the gradual expression of alate characters (Nijhout and Wheeler 1982).

The elevated JH levels in *C. secundus* larvae, contrasting with the picture obtained for the cockroach *N. cinerea* (Lanzrein et al. 1985b), have led us to propose that the larval stage of termites may be equivalent to the pronymphal stage of cockroaches. In support of this idea are not only the JH levels at the larval/nymphal transition, but also the JH and ecdysteroid titers in the final nymphal instar, which are reminiscent of those of fifth instar *Manduca sexta* larvae, the endocrine model system for holometabolous development. The high degree of plasticity in caste development of termites, especially in the lower termites, may thus have its evolutionary origins in a de-embryonization process similar to holometabolans, yet in an isolated taxon within the cockroach clade. Furthermore, termites seem to have fully exploited the endocrine signature underlying the pronymphal/larval transition (Truman and Riddiford 1999), so that their developmental trajectories include not only progressive and stationary, but even regressive molts. The latter, obviously, only make sense in a social context.

Acknowledgments

We thank Walter Goodman and Walter Bollenbacher for donating, respectively, a JH and an ecdysone antiserum for use in the radioimmunoassays. Financial support was provided by a bilateral CAPES/DAAD grant (PROBRAL 261/07) and a research grant by the Deutsche Forschungsgemeinschaft (DFG, KO 1895/6).

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