

Research article

Caste differentiation responses of two sympatric *Reticulitermes* termite species to juvenile hormone homologs and synthetic juvenoids in two laboratory assays

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Summary. We report on our investigations comparing three juvenile hormone (JH) homologs and two synthetic juvenoids to induce caste differentiation in laboratory colonies of *Reticulitermes flavipes* and *R. tibialis*. Two laboratory assays were evaluated as model systems for inducing caste differentiation: (1) shorter-term dish assays on groups of 20 individuals and (2) longer-term feeding assays on groups of 500 individuals. Each assay possessed attributes that can be considered advantageous under certain conditions. Specifically, dish assays were most suitable for presoldier and soldier induction, while jar assays provided for the induction of nymphs, presoldiers, soldiers, neotenic reproductives, and intercastes. Differences in response to the JH homologs and synthetic juvenoids were noted between species, suggesting differences in JH physiology may exist between *R. flavipes* and *R. tibialis*. Substantial morphological impacts were noted in association with some treatments, including (1) juvenoid-induced mandibular malformation in presoldiers, (2) JH II-induced abdominal elongation in *R. flavipes* soldiers and workers (associated with a presence of internal reproductive anatomy that is consistent with what would be expected to occur in pseudergates), and (3) JH II-induced soldier-nymph intercastes in *R. tibialis* that were able to further molt into soldier-alate intercastes. Findings are discussed in relation to the potential differences in JH-related physiology between *R. flavipes* and *R. tibialis*, and the use of model systems to induce rare castes and intercastes for molecular investigations of caste differentiation.

Key words: Isoptera, Rhinotermitidae, pyriproxyfen, hydro-prene, intercaste.

Introduction

Termites are social insects with a dual economic and ecological significance. The combined global economic impact of termite feeding on buildings and structures, plus control costs, is estimated to exceed US \$ 20 billion annually (Su, 2002). At the same time, however, termites serve highly important ecological functions because of their role in the decomposition of cellulose materials in natural ecosystems (Sugimoto et al., 2000). In the U.S., the primary termite species to occur are from the genus *Reticulitermes*. In the Midwestern U.S., the two most commonly occurring *Reticulitermes* species are *R. flavipes* (Kollar) and *R. tibialis* Banks (Snyder, 1954; Weesner, 1965; Nutting, 1990). Much remains unknown with respect to the basic biology of termites (e.g., molecular basis of caste differentiation, reproductive strategies, etc.), and relating to the potential mechanisms that may be in place to partition sympatric species. This is especially true for *R. flavipes* and *R. tibialis*, for which very little basic biology and ecology are defined. Recent molecular systematic research indicates that *R. tibialis* and *R. flavipes* are phylogenetically distant members of the genus *Reticulitermes* (Austin et al., 2002; Ye et al., 2003). It would therefore be expected that significant and non-trivial biological differences between these two species should exist.

Termites, including those of the genus *Reticulitermes*, undergo a caste differentiation process that is unparalleled among social insects (Wilson, 1971). There are three termite castes, which are polyphenic forms that perform specific colony functions: workers, reproductives and soldiers. The worker caste is responsible for foraging and brood tending; reproductives (multiple forms) are responsible for the production of offspring; and soldiers provide colony defense and intra-colonial JH regulation through several potential physiological mechanisms (Henderson, 1998). With respect to

caste differentiation in the *lower* termites (including Rhinotermitidae), the soldiers and reproductives represent developmental end points (Buchli, 1958; Noirot, 1982; Thorne, 1996; Roisin, 2000).

The worker caste in lower termites is composed of several juvenile instars that can differentiate along the presoldier/soldier route, or remain un-differentiated through successive instars in the absence of relevant stimuli. Nymphs proceed through several instars before they emerge as adult reproductives (either alates or second-form neotenic reproductives); while a single presoldier instar is directly followed by emergence of the soldier. Because soldiers retain prothoracic glands, they are not considered adult (Thorne, 1996). Nymphs and last-instar larvae destined to differentiate along the imaginal route may also undergo regressive molts into pseudergate workers (Buchli, 1958), although the pseudergate stage is considered rare in *Reticulitermes* (Thorne, 1996).

In termites, juvenile hormone (JH) is central to caste differentiation. When its titers occur above specific intra-colony thresholds, JH induces the differentiation of a presoldier instar from workers, while at the same time, suppresses differentiation of nymphs from last instar larvae. It is only when JH titers drop below specific thresholds that nymphal differentiation is thought to occur (reviewed by Henderson, 1998). In non-Isopteran insects, JH expression is directly linked to the maintenance of immature features in juveniles and gonadal maturation in adults (Gilbert et al., 2000). In honey bees, JH expression is also associated with temporal polyethism in workers (Schulz et al., 2002). The dominant JH homolog in insects is JH III (Baker, 1990; Gilbert et al., 2000), which has also been identified in a small number of termites (Meyer et al., 1976; Lanzrein et al., 1982; Greenberg and Tobe, 1985). Other JH homologs have been identified in higher insects, such as the Lepidoptera, where at least four homologs are synthesized (JH 0, I, II and III; Gilbert et al., 2000). No verification exists regarding the actual form of JH that is utilized in the genus *Reticulitermes*, nor if there are multiple forms with differing functions, or variability among *Reticulitermes* species.

The ability of synthetic JH, crude JH mixtures, and synthetic juvenoids to induce presoldier differentiation from *Reticulitermes* workers is well established (e.g., Howard and Haverty, 1979; Hrdý, 1982; Jones, 1984; Su and Scheffrahn, 1989; Okot-Kotber et al., 1991; Lelis and Everaerts, 1993; Hrdý et al., 2001). To our knowledge, only two reports exist of the effects of pure juvenile hormone homologs on a *Reticulitermes* termite (JH II and JH III; reviewed by Hrdý, 1982); however, these investigations exclusively focused on presoldier induction in European *R. santonensis*. Furthermore, caste differentiation in *R. tibialis* has never been investigated. Using laboratory colonies of two sympatric *Reticulitermes* species from the U.S., the objectives of these studies were to: (1) develop baseline information on the effects of JH homologs (JH I, II and III) and two synthetic juvenoids (pyriproxyfen and hydroprene) on caste differentiation; (2) develop baseline information on the highly under-studied U.S. species *R. tibialis*; and (3) evaluate two model laborato-

ry assays for their relative ability to induce caste differentiation in laboratory colonies of *R. flavipes* and *R. tibialis*.

Materials and methods

Termites

R. flavipes and *R. tibialis* individuals were collected in Tippecanoe County, Indiana (U.S.A.), and held in the laboratory for 2–4 weeks before use in assays. Laboratory colonies were initiated from the collections, which included workers, nymphs (and possibly pseudergates), presoldiers, soldiers, and neotenic reproductives. *R. flavipes* were collected from a Purdue University-Entomological field research station ('EFOB') and *R. tibialis* from a site on the Purdue University campus. Collections of each species were made from a number of locations over expansive geographic areas (approx. 2–3 ha.), and no intra-specific aggressive behavior was observed between individuals from different collection points. Identity of the respective termite species was verified by external morphology (Nutting, 1990), and by DNA sequence of the mitochondrial cytochrome oxidase subunit II gene (Ye et al., 2003; Genbank Accession Nos. AY168209 and AY168206). Both species are well documented in the area where collections were made (i.e., central Indiana; Nutting, 1990). Termites were collected within buried cardboard rolls, brought into the laboratory, and allowed to migrate to moistened wood as the cardboard dried under ambient conditions. In laboratory colonies, several thousand termites were maintained within individual sealed plastic containers (25–27°C, > 90% RH). They were provided moistened pine blocks, tongue depressors, and laboratory paper towels for food and harborage. Moisture in the form of reverse-osmosis water was provided judiciously as needed with a spray bottle. Fifth through seventh instar workers from independent rearing containers were used in individual assay replicates.

Chemicals

Test chemicals included synthetic JH I (racemic mixture; 78% purity; SciTech Inc., Czech Republic), JH II (racemic mixture; 78% purity; SciTech Inc.), synthetic JH III (*trans* isomer; 75% purity, Sigma Inc.), pyriproxyfen (98.2% purity; MGK Inc.), and hydroprene (98.2% AI; Zoecon Inc.). Most impurities occurring with the synthetic JH homologs are JH-acid and JH-diol metabolites of the respective JH forms (Sci-Tech Inc., pers. comm.). Stock solutions of 5 mg/ml were prepared in acetone (Sigma analytical grade) for all test chemicals, and four serially diluted concentrations plus an acetone control were tested.

Dish assays

Dish assays were modeled after the assay described by Okot-Kotber et al. (1991). Paired filter paper sandwiches (3.5 cm diam; Whatman #1) were treated with 300 µl of each JH homolog or juvenoid dilution to provide 300, 150, 75, and 37.5 µg test chemical per assay dish. Each filter paper weighed approximately 75 mg. After acetone had evaporated, the filter papers were wetted with 100 µl water and placed in 5 cm Petri plates with 20 worker termites (5th–7th instar) and a cotton-plugged Eppendorf tube (0.5 ml size) containing ca. 0.3 ml water. Termites were considered to be workers if they did not possess any sign of wing buds or distended abdomens, as would be present in pseudergates or nymphs (Buchli, 1958). Plates were not sealed, but held closed with two narrow pieces of tape at opposite sides of the dishes. The plates were held in complete darkness at room temperature (25–27°C), within sealed plastic containers, and with wetted paper towels (to maintain high humidity). Assays were replicated five times per treatment, and lasted 15 days. Cumulative mortality and presoldier formation were scored every fifth day. Both workers and presoldiers were scored for mortality. Presoldier formation and mortality data were analyzed by a mean separation test

that used a comparison-wise error calculation (the Ryan-Q F test; SAS Institute, 2001).

Jar feeding assays

Jar feeding assays were modeled after the system described by Haverty (1979). Individual glass jars (500 ml volume) received 150 ml of sand, 150 ml of vermiculite, and 100 ml of water. The contents were mixed thoroughly and 500 termites were added per jar. One-half of the jars received 100% worker termites, and one-half received 98% workers (5th–7th instar) plus 2% soldiers. Termites were considered to be workers if they did not possess any sign of wing buds or distended abdomens, as would be present in pseudergates or nymphs (Buchli, 1958). After one day, individual jars received single filter papers (9 cm diam; Whatman #1) treated with various test chemical concentrations, or acetone (for controls). Each filter paper weighed approximately 530 mg. Test chemicals were provided in quantities pre-determined to elicit minimal mortality in *R. flavipes* feeding assays (JH III = 150 µg; JH II = 75 µg; pyriproxyfen = 100 µg; hydroprene = 200 µg). Each treatment was replicated three times and in duplicate between soldier and no-soldier experiments. Jars were held in darkness at 25–27°C. Filter papers were replaced weekly to ensure maximal availability of test chemicals. After either 6 or 12 weeks (*R. tibialis* = 6 weeks; *R. flavipes* = 12 weeks), assays were terminated and scored. From raw data, the following percentages were determined: overall survival, worker, presoldier and soldier, nymph, and neotenic reproductive. Data by species were analyzed by a mean separation test that used a comparison-wise error calculation (the Ryan-Q F test; SAS Institute, 2001).

Results

Dish assays

Figure 1 shows results for dish assays. Although mortality was observed in some control (acetone-treated) bioassays, it was never significantly different from zero ($\alpha = 0.05$). In addition, presoldier production was never observed in control assays. JH I elicited high toxicity to both species at all test concentrations, and only one presoldier was observed throughout the JH I assays (*R. tibialis* at 75 µg). JH II and III were less toxic than JH I, and were significantly more effective at inducing presoldier differentiation from workers than the juvenoids. JH II induced greater presoldier differentiation in *R. flavipes* but higher mortality in *R. tibialis*. JH III induced greater presoldier differentiation in *R. tibialis* than *R. flavipes*. With the two juvenoids pyriproxyfen and hydroprene, no concentration-dependent effects in either presoldier induction or mortality were observed for *R. flavipes*. There was no significant juvenoid concentration-dependence of presoldier differentiation in *R. tibialis*, but there were significant concentration-dependent effects of the juvenoids on *R. tibialis* mortality.

Jar feeding assays

Jar assays were conducted in order to determine longer term effects of test chemicals on larger groups of termites (relative to dish assays). Results for *R. flavipes* and *R. tibialis* jar assays are shown in Tables 1 and 2, respectively. Optimal times of 6 and 12 weeks were chosen for evaluation between

the two species (*R. tibialis* and *R. flavipes*) because these times were associated with significant biological effects and the lowest possible mortality (results not shown). Control survival was greatest for *R. flavipes*, but was enhanced in both species by the inclusion of soldiers at the beginning of assays. In the majority of treatments, both species had significantly enhanced survival in the presence of soldiers. The most dramatic soldier-effect on survival was in *R. tibialis* pyriproxyfen assays, where 81.2% and 0% survival occurred in assays with and without soldiers included at the beginning of assays, respectively. Several female third-form reproductives were observed in *R. tibialis* control assays and *R. flavipes* JH II assays. These third-form reproductives did not possess wing pads and were eyeless (Fig. 2F).

Ending worker proportions were above 95% and were not significantly different between all treatments except those involving *R. tibialis* and juvenoids, in which worker percentages were significantly reduced to 30.8% and 86.3%, respectively, for pyriproxyfen and hydroprene. Combined soldier and presoldier percentages were near expected natural levels for all treatments, except in *R. tibialis* pyriproxyfen assays where soldier/presoldier levels composed 69.2% of surviving termites. Finally, in *R. tibialis* JH II jar assays, 100% of individuals scored as soldiers were either soldier-nymph (Fig. 2K, L, N) or soldier-alate (Fig. 2P, Q) intercastes. These intercaste individuals were scored as soldiers due to the fact that apparently normal nymphs were observed in the same assays, and because of additional morphological characteristics noted below. Although some effects on reproductive anatomy were identified, no *R. tibialis*-analogous effects were observed in *R. flavipes* soldiers or presoldiers in response to JH II exposure (see below).

Morphological observations

Individuals shown in Figure 2 were either obtained from laboratory colonies or jar feeding assays. JH III treatment led to apparently normal morphological features in presoldiers and other castes, which were not discernable from controls (Fig. 2A–2D). Pyriproxyfen and hydroprene treatment was associated with increased mandibular melanization in presoldiers (compare Fig. 2C vs. 2G) and soldiers with shortened or otherwise qualitatively malformed mandibles. In *R. flavipes*, JH II treatment was associated with highly pronounced abdominal distention in ca. 10% of surviving soldiers and workers (Fig. 2H, 2I), and decreased cuticular melanization of late instar nymphs (Fig. 2J). Morphometric measurements on JH II-exposed workers with distended abdomens revealed a significant 1.48-fold increase in abdominal length relative to untreated workers (paired *t*-test, $n = 22$, $P \leq 0.01$). In the *R. flavipes* JH II-exposed workers and soldiers with distended abdomens, dissections revealed the presence of developing reproductive anatomy that is consistent with what would be expected in a pseudergate individual (Fig. 2M, 2O). In *R. tibialis*, JH II treatment was associated with formation of soldier-nymph and soldier-alate intercastes (Fig. 2K, 2L, 2N, 2P, 2Q). These intercastes also possessed mandibular

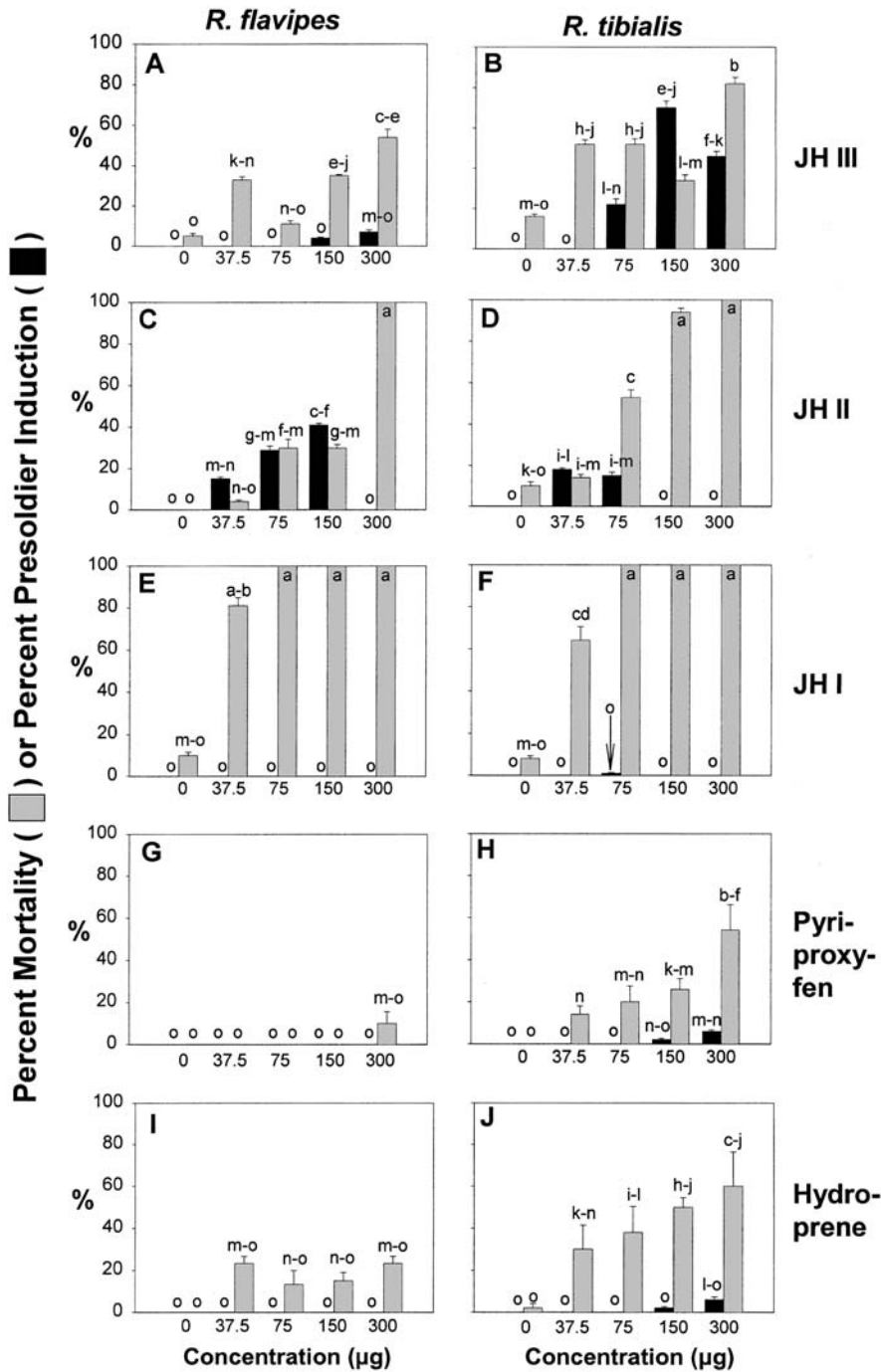


Figure 1. Average presoldier induction (black bars) and mortality (gray bars) in dish assays, as induced by various JH homologs and synthetic juvenoids: (A–B) JH III, (C–D) JH II, (E–F) JH I, (G–H) pyriproxyfen, and (I–J) hydro-prene. *R. flavipes* and *R. tibialis* results are shown in the left and right columns, respectively. Several concentrations and a control were tested for each species and chemical. Assays were initiated with twenty worker termites, and results are the average ± std. error of five replicated assays at 15 days. Means with the same letters are not significantly different by the Ryan-Q test ($\alpha = 0.05$, $df = 391$, $MSE = 88.8$)

Table 1. Survival (Avg. % \pm SE) of various *R. flavipes* castes and developmental stages after 12 week jar-feeding assays. Three replicate assays per treatment were initiated with either 500 5th–7th instar workers, or 98% workers plus 2% soldiers. Filter papers treated with indicated quantities of JH homologs or synthetic juvenoids were replaced in jars weekly. Values followed by the same letter are not significantly different by the Ryan-Q (F) test ($p < 0.05$, $df = 100$, $MSE = 12.4$)

Species	Treatment ($\mu\text{g}/\text{assay}$)	Soldiers Added	% Overall Survival	% of Various Castes and Forms			
				Worker ^a	Soldier ^b	Nymph ^c	Third-Form Reproductive ^d
<i>R. flavipes</i>	Acetone	No	67.1 \pm 5.9 b	97.0 \pm 0.6 a	2.7 \pm 0.6 e	0.3 \pm 0.2 e	0.0 \pm 0.0 f
		Yes	71.8 \pm 2.0 b	96.2 \pm 0.2 a	3.9 \pm 0.5 e	0.1 \pm 0.1 ef	0.0 \pm 0.0 f
	JH II [75 μg]	No	39.5 \pm 2.5 d	96.3 \pm 1.0 a	3.3 \pm 0.6 e	0.4 \pm 0.3 e	0.1 \pm 0.0 ef
		Yes	46.9 \pm 4.7 c	95.5 \pm 0.2 a	3.0 \pm 0.1 e	1.5 \pm 0.1 e	0.0 \pm 0.0 f
	JH III [150 μg]	No	64.9 \pm 6.5 b	95.6 \pm 0.4 a	3.5 \pm 0.6 e	0.9 \pm 0.2 e	0.0 \pm 0.0 f
		Yes	68.9 \pm 6.7 b	95.0 \pm 1.0 a	4.9 \pm 1.0 e	0.2 \pm 0.1 e	0.0 \pm 0.0 f
	Pyriproxyfen [100 μg]	No	53.5 \pm 3.2 c	95.7 \pm 0.9 a	3.8 \pm 0.9 e	0.5 \pm 0.1 e	0.0 \pm 0.0 f
		Yes	69.4 \pm 2.3 b	96.5 \pm 0.7 a	3.2 \pm 0.5 e	0.4 \pm 0.2 e	0.0 \pm 0.0 f
	Hydroprene [200 μg]	No	51.3 \pm 7.3 c	96.2 \pm 0.6 a	3.5 \pm 0.8 e	0.3 \pm 0.2 ef	0.0 \pm 0.0 f
		Yes	73.2 \pm 2.7 b	97.0 \pm 0.2 a	3.0 \pm 0.2 e	0.0 \pm 0.0 f	0.0 \pm 0.0 f

^a Wing pads absent; soldier/presoldier characters absent.

^b Soldier/presoldier characters present.

^c Wing pads present; soldier/presoldier characters absent.

^d Distended abdomen; wing pads absent; eyeless.

Table 2. Survival (Avg. % \pm SE) of various *R. tibialis* castes and developmental stages after 6 week jar-feeding assays. Three replicate assays per treatment were initiated with either 500 5th–7th instar workers, or 98% workers plus 2% soldiers. Filter papers treated with indicated quantities of JH homologs or synthetic juvenoids were replaced in jars weekly. Values followed by the same letter are not significantly different by the Ryan-Q (F) test ($p < 0.05$, $df = 100$, $MSE = 7.3$). Values enclosed in box represent 100% soldier-nymph and soldier-alate intercastes

Species	Treatment ($\mu\text{g}/\text{assay}$)	Soldiers Added	% Overall Survival	% of Various Castes and Forms			
				Worker ^a	Soldier ^b	Nymph ^c	Third-Form Reproductive ^d
<i>R. tibialis</i>	Acetone	No	27.8 \pm 19.7 hi	96.6 \pm 22.8 bcd	0.0 \pm 0.0 k	0.0 \pm 0.0 k	1.2 \pm 0.8 j
		Yes	93.3 \pm 0.4 abcd	97.0 \pm 0.5 abc	2.6 \pm 0.5 j	0.0 \pm 0.0 k	0.0 \pm 0.0 k
	JH II [75 μg]	No	89.1 \pm 1.1 cde	95.6 \pm 2.4 abc	3.5 \pm 1.9 j	0.9 \pm 0.1 j	0.0 \pm 0.0 k
		Yes	93.2 \pm 1.5 abcd	96.2 \pm 1.9 abc	3.2 \pm 1.9 j	0.6 \pm 0.1 j	0.0 \pm 0.0 k
	JH III [150 μg]	No	40.9 \pm 9.8 h	99.6 \pm 0.2 a	0.4 \pm 0.2 jk	0.0 \pm 0.0 k	0.0 \pm 0.0 k
		Yes	87.1 \pm 7.1 def	96.1 \pm 1.3 abc	3.6 \pm 1.4 j	0.3 \pm 0.2 jk	0.0 \pm 0.0 k
	Pyriproxyfen [100 μg]	No	0.0 \pm 0.0 k	0.0 \pm 0.0 k	0.0 \pm 0.0 k	0.0 \pm 0.0 k	0.0 \pm 0.0 k
		Yes	81.2 \pm 2.8 f	30.8 \pm 4.7 i	69.2 \pm 4.7 g	0.0 \pm 0.0 k	0.0 \pm 0.0 k
	Hydroprene [200 μg]	No	64.7 \pm 1.7 g	98.8 \pm 0.2 ab	1.2 \pm 0.2 j	0.0 \pm 0.0 k	0.0 \pm 0.0 k
		Yes	27.1 \pm 23.5 hi	86.3 \pm 27.8 ef	1.0 \pm 0.9 jk	0.3 \pm 0.2 jk	0.0 \pm 0.0 k

^a Wing pads absent; soldier/presoldier characters absent.

^b Soldier/presoldier characters present.

^c Wing pads present; soldier/presoldier characters absent.

^d Distended abdomen; wing pads absent; eyeless.

and wing malformations (Fig. 4P), as well as malformed legs (Fig. 2Q).

In both species, there was a succession of intercaste developmental events in association with JH II exposure, which was evident based on the presence of apparently successive developmental forms at the end of jar assays. In the *R. flavipes* JH II intercastes, developmental events appeared to involve: (1) differentiation of workers with distended

abdomens and developing internal reproductive anatomy (i.e., a pseudergate-like individual), (2) differentiation of apparent pseudergate-presoldiers, then (3) emergence of apparent pseudergate-soldiers. In the *R. tibialis* JH II intercastes, these developmental events appeared to involve: (1) differentiation of presoldiers with internal reproductive anatomy, (2) eclosion of soldiers with external nymphal features, and finally (3) eclosion of soldier-imago intercastes.

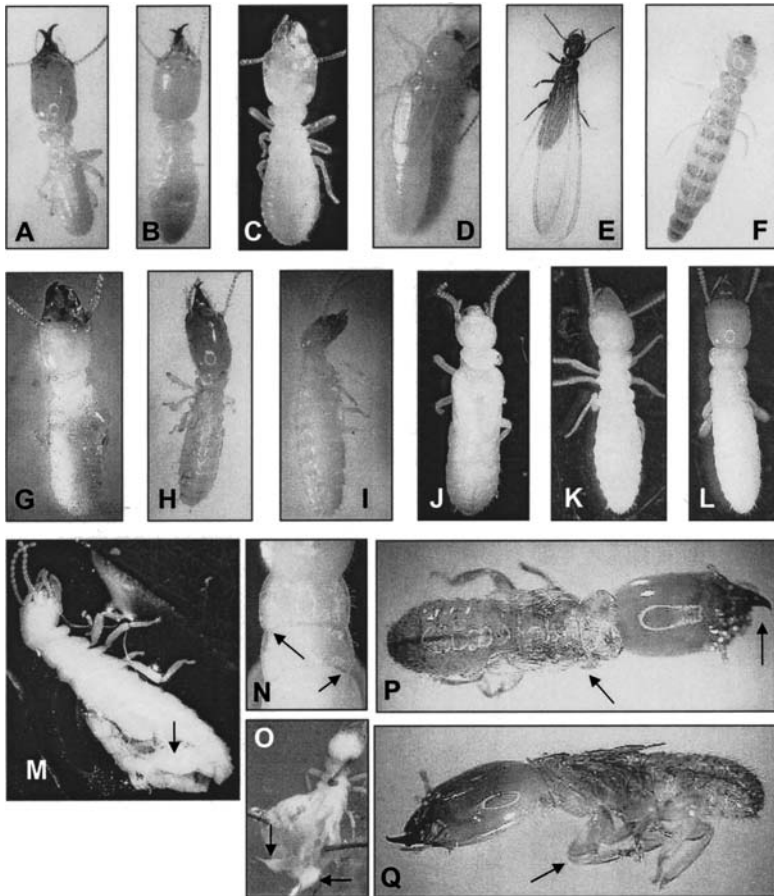


Figure 2. Morphological impacts on *Reticulitermes* termites by JH homologs and synthetic juvenoids in jar feeding assays. (A) normal *R. tibialis* soldier, (B) normal *R. flavipes* soldier, (C) normal *R. tibialis* presoldier, (D) normal *R. tibialis* late instar nymph, (E) normal *R. tibialis* alate, (F) normal *R. tibialis* third-form reproductive, (G) *R. tibialis* presoldier showing pyriproxyfen effects (note mandibular defects), (H) *R. flavipes* 'pseudergate' soldier showing JH II effects (note lengthened abdomen), (I) *R. flavipes* pseudergate-like worker showing JH II effects (note lengthened abdomen), (J) *R. flavipes* nymph showing JH II effects (note lack of melanization), (K) *R. tibialis* presoldier showing JH II effects (note shortened mandibles and worker-like thorax), (L) *R. tibialis* soldier-nymph intercaste showing JH II effects (note sclerotized head and initiation of wing buds; see N for magnified view), (M) developing internal reproductive anatomy in pseudergate-like *R. flavipes* shown in I, (N) magnified view of wing buds on *R. tibialis* JH II soldier-nymph intercaste shown in L, (O) developing reproductive anatomy in *R. tibialis* soldier-nymph shown in L, (P–Q) top and side views, respectively, of *R. tibialis* JH II soldier-imago intercaste (note shortened mandibles, malformed wings, and characteristic leg curvature)

Discussion

Use of JH homologs to induce caste differentiation

In dish assays modeled after Okot-Kotber et al. (1991), the JH homologs JH II and JH III were effective at inducing presoldier differentiation from both *R. flavipes* and *R. tibialis* workers, although JH III appeared more effective on *R. tibialis*. JH I, alternatively, was highly toxic to worker termites irrespective of species at all concentrations tested. Longer term effects of JH II and III at inducing presoldier differentiation in jar-feeding assays (modeled after Haverty, 1979) were not as pronounced as in dish assays. It is not known if these differences relate to differential presoldier/soldier survival between the assays; however, in the feeding assays, presoldier and soldier levels were consistent with what would be expected in natural field colonies (Haverty, 1977). In this regard, there were no significant differences between treatments and controls.

Differentiation of nymph and third-form reproductive phenotypes from workers was also observed in the longer-term feeding assays. Relative to dish assays, this is presumably a result of the longer assay time, the greater number of workers included at the initiation of assays (n = 500 vs. 20), and a corresponding dilution of available JH quantities via a group effect (i. e., JH is capable of being transferred between

individuals; Henderson, 1998). JH I was examined preliminarily in feeding assays (37.5 µg per assay). However, as with shorter-term contact assays, high-level mortality had occurred so rapidly that no caste-differentiation effects could be observed (results not shown). This result is not completely unexpected, as some synthetic JH analogs have previously shown high-level acute toxicity in *Reticulitermes* (Hrdý, 1982). Our goal was to compare the relative effects of JH I, II, and III across an identical range of concentrations; therefore, we did not further test JH I at lower concentrations. It is possible that JH I could induce caste differentiation effects at lower concentrations, and this possibility should be considered in future research.

In the feeding assays, substantial differences were also observed between each species with the JH homologs II and III. In both *R. flavipes* and *R. tibialis*, JH III treatments led to the production of apparently normal presoldiers, soldiers and nymphs. However, JH II had very different effects. In *R. flavipes*, JH II treatments were associated with lengthened abdomens and developing internal reproductive anatomy in a portion of workers, presoldiers and soldiers. The soldiers, which possessed internal reproductive anatomy, had apparently normal heads and thoraces, and no signs of wing buds. In *R. flavipes* nymphs, a decreased level of melanization was also observed in association with JH II treatments. This effect is not readily explained, except that cuticular melanization

effects have also been noted in association with some juvenoids (e.g., Zufelato et al., 2000). None of the effects noted above were reported in direct contact assays in association with JH treatment of the closely related species *R. l. santonensis* (Hrdý, 1982), which is now considered a synonym of *R. flavipes* (e.g., Austin et al., 2002).

JH II impacts on *R. tibialis* were markedly different from *R. flavipes*. In jar assays, apparently normal nymphs were observed, while the only individuals displaying presoldier and soldier features were actually soldier-nymph and soldier-alate intercastes. Interestingly, from the 1st thoracic segment forward these intercaste individuals possessed features consistent with presoldiers or soldiers, while they possessed nymphal characters posterior to the second thoracic segment after the presoldier-to-soldier molt. In addition, a very pronounced curvature of the legs was observed in these *R. tibialis* intercastes, which was first apparent in the soldier-nymph instar. Soldier imagos (i.e., reproductive soldiers) are well documented in some primitive termite taxa (Thorne, 1997); however, Buchli (1958) observed nymph-soldier intercastes in *R. l. santonensis*. We hypothesize that the individuals we observed were true soldier-nymphs, and not nymph-soldiers with origins along the imaginal differentiation route (as described by Buchli, 1958). This hypothesis is based on the following observations: (1) workers (not pseudergates or nymphs) were used to initiate assays, (2) apparently normal nymphs were also observed, and (3) nymphal/imaginal features developed after soldier features (see Fig. 2). The production of adult soldier-imago intercastes has only been documented once previously in a *Reticulitermes* species, in response to juvenoid treatment (Jones, 1984). In this case, soldier-alate intercastes were observed following treatment of *R. virginicus* workers with the fenoxycarb-related juvenoid Ro 16-1295.

Differential juvenoid effects between R. tibialis and R. flavipes

Although juvenoid effects never have been examined on *R. tibialis*, the ability of synthetic juvenoids to induce presoldier differentiation from workers of other *Reticulitermes* species has long been known (e.g., Howard and Haverty, 1979; Hrdý, 1982; Jones, 1984; Su and Scheffrahn, 1989; Okot-Kotber et al., 1991; Lelis and Everaerts, 1993; Hrdý et al., 2001). In the present study, we examined the synthetic juvenoids hydroprene and pyriproxyfen at concentration levels on the same order of magnitude as previously reported in the literature (e.g., Su and Scheffrahn, 1989). The two juvenoids tested here were structurally very different from each other. The structure of hydroprene generally resembles a typical JH 'backbone', while pyriproxyfen bears no superficial resemblance to JH.

The two juvenoids were associated with similar morphological effects on both *R. flavipes* and *R. tibialis*. These effects included increased mandibular melanization and other variable mandibular defects. Previous studies have noted similar effects in *Reticulitermes* termites (Jones, 1984; Lelis

and Everaerts, 1993), and honey bees (Zufelato et al., 2000) after exposure to juvenoids. The mandibular abnormalities we have observed are consistent with what has been described as being present in 'presoldier-soldier intercastes' (Jones, 1984; Lelis and Everaerts, 1993). However, we observed no juvenoid-induced soldier-alate or soldier-nymph intercastes as noted above for JH II, or as noted for the fenoxycarb-related juvenoid Ro 16-1295 by Jones (1984). Other researchers have likewise not reported juvenoid effects on the formation of soldier-imaginal intercastes in *Reticulitermes* termites (e.g., Okot-Kotber et al., 1991; Lelis and Everaerts, 1993).

R. tibialis was greatly impacted by the juvenoids in assays that were shorter than those assays used for *R. flavipes* (6 vs. 12 wk). In *R. tibialis* pyriproxyfen assays where soldiers were added at the onset, there was 81.2% survival with 69.2% soldier/presoldier formation in survivors. This effect was not observed in pyriproxyfen assays in which no soldiers were added, and further, significantly greater mortality was also observed in these assays (i.e., 100% mortality). These exclusive effects by pyriproxyfen on high-level presoldier induction in *R. tibialis* suggest that differences in JH physiology may exist between *R. tibialis* and *R. flavipes*. This conclusion is in agreement with phylogenetic research that has shown *R. tibialis* and *R. flavipes* to be phylogenetically distant members of the *Reticulitermes* genus (Austin et al., 2002; Ye et al., 2003), and our own unpublished observations which suggest clear ecological differences between these two species. JH physiology and soldier differentiation thresholds are closely linked to defensive and aggression characteristics of individual termite species (Lenz, 1976). Such differences in JH physiology, therefore, may prove to be highly informative with respect to explaining basic ecological differences between termite species.

Conclusions

Only two previous studies have examined JH homologs on *Reticulitermes* termites (reviewed by Hrdý, 1982). Because these previous studies only examined presoldier induction in short-term contact assays, no observations were available until now relative to the effects of these materials on differentiation of other castes or phenotypes. We have demonstrated impacts by relatively pure JH on both small groups of workers and larger simulated colonial groups. The fact that the JH homologs could not induce higher numbers of presoldiers and soldiers is in agreement with previous researcher conclusions that complex feedback mechanisms exist, which serve to precisely regulate presoldier/soldier differentiation (Noirot, 1982; Henderson, 1998). In this regard, the assays described here could be used in further research to examine the effects of reproductives, seasonality, and nutritional status on JH-induced caste differentiation, as well as other potential configurations that may elucidate feedback mechanisms.

It is clear from our results that both JH II and JH III have effects on *Reticulitermes* caste differentiation which favor

their experimental use in the two assay systems that we evaluated. Because the juvenoids were associated with morphological abnormalities, they may not be as useful as the JH homologs in morphometric studies, or assays that are coupled with molecular investigation of *Reticulitermes* caste differentiation. This conclusion is supported by previous results obtained by Lelis and Everaerts (1993) who documented extensive morphological abnormalities in *R. santonensis* exposed to the juvenoid methoprene, and Miura et al. (2003) who noted morphological abnormalities in *Zootermopsis nevadensis* alates by the juvenoid pyriproxyfen. However, Koshikawa et al. (2002) reported that juvenoid-induced presoldiers were morphologically identical to natural presoldiers in *Hodotermopsis japonica*, indicating that juvenoids may reliably be substituted for JH homologs in morphometric and molecular studies on some termite taxa.

With respect to the model assays, dish assays are most suitable for the induction of presoldier and soldier differentiation, while jar assays provide for the induction of nymphal, presoldier, soldier and neotenic reproductive differentiation. Depending on whether or not intercastes are desired, either JH II or JH III (respectively) can be used. These assays have already been utilized in combination with molecular-genomic research to discover developmental and regulatory genes (Wu-Scharf et al., 2003) and to define the expression levels of genes in presoldiers, soldiers, workers and nymphs/pseudergates (Scharf et al., 2003).

Much like the reliance on mutations to unravel biological, molecular, and genetic processes in *Drosophila* (Rubin, 1988), molecular studies of rare castes and intercastes in termites may be useful in addressing long-standing uncertainties relating to termite caste differentiation. Therefore, having reliable caste-differentiation assays, such as those evaluated here, will be highly important in ongoing and future studies on the molecular processes involved in *Reticulitermes* caste differentiation.

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