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Endocrine effects of social stimuli on maturing queens of the dampwood termite *Zootermopsis angusticollis*

COLIN S. BRENT[1], COBY SCHAL[2] and EDWARD L. VARGO[2]
[1]School of Life Sciences, Arizona State University, Tempe, AZ, U.S.A. and [2]Department of Entomology and W. M. Keck Center for Behavioural Biology, North Carolina State University, Raleigh, NC, U.S.A.

Abstract. The reproductive physiology of social insects is often highly responsive to social stimuli from nestmates, but the mechanisms underlying this sensitivity are usually poorly understood. The effect of varied social conditions on the endocrinology and ovarian maturation in maturing female primary reproductives of the dampwood termite *Zootermopsis angusticollis* Hagen is studied to better understand their developmental responses to conspecifics. Newly dealate queens are paired with a reproductive male, with another maturing female, or are allowed to mature in isolation. Developmental responses are tracked by monitoring rates of juvenile hormone (JH) production by corpora allata *in vitro*, haemolymph ecdysteroids titres and ovarian development after 5, 10, 15 and 30 days of maturation. Significant declines in rates of JH production rates are observed by days 5, 10 and 30, respectively, for females paired with males, left to mature in isolation or paired with a female. Ecdysteroid titres increase by day 5 and stay elevated in females nesting with males. By contrast, for CA from females that are isolated or paired with another female, the titres decline and stay low until at least day 15, but increase significantly by day 30. These hormonal differences correspond to differential rates of physiological maturation, with more rapid ovarian development occurring in females paired with a male, than in those nesting alone or with another female. The results suggest that JH and ecdysteroids modulate the ovarian response of alates to stimuli from nestmates during this period of maturation, and that JH and ecdysteroid titres may be regulated independently during this period of development.

Key words. Ecdysteroids, Isoptera, juvenile hormone, reproductive maturation, queen.

Introduction

Stimuli produced by social insects can have profound effects upon the course, timing and extent of the development of nestmates, especially during critical periods of maturation. In some species, a nestmate's development can be manipulated by specific behaviours (Spradbery, 1965; West-Eberhard, 1969, 1978; Cole, 1981; Zimmerman, 1983; Michener, 1990; Spradbery, 1991; Packer, 1993; Cuvillier-Hot *et al.*, 2004a,b) whereas in others such manipulation is achieved though the release of pheromones or a combination of behavioural and pheromonal interactions (Lüscher, 1972; Röseler *et al.*, 1981; Fletcher & Blum, 1983; Zimmerman, 1983; Bordereau, 1985; Fletcher & Ross, 1985; Röseler, 1991; Spradbury, 1991; Keller & Nonacs, 1993; Keller & Vargo, 1993; Vargo & Laurel, 1994; Sommer & Hölldobler, 1995). Because the ability to manipulate the reproductive capability of nestmates may be a key step to the evolution of castes and eusociality (West-Eberhard, 1987, 1996), identifying the regulatory mechanisms through which these stimuli influence reproduction is essential to determining how insect societies function and are maintained.

Among the 'primitive' Lower termites, in which all individuals maintain some degree of developmental totipotency throughout their lives (Miller, 1969; Noirot, 1985; Shellman-Reeve, 1997; Thorne, 1997; Thorne *et al.*, 1999), stimuli from nestmates have been shown to be crucial in regulating

Correspondence: C. S. Brent, School of Life Sciences, Arizona State University, Box 874501, Tempe, Arizona 85287-4501, U.S.A. Tel.: +1 480 9657292; fax: +1 480 9656899; e-mail: colin.brent@asu.edu

both caste differentiation and reproductive activity (Noirot, 1985). The dampwood termite *Zootermopsis angusticollis* Hagen, a Lower termite, is highly responsive to stimuli from a number of different nestmate castes (Castle, 1934; Light, 1943; Light & Weesner, 1951; Greenberg & Stuart, 1979, 1982; Brent & Traniello, 2001a, b, c). Of particular interest are the social stimuli, which can affect individual fecundity, because these maintain the caste system that limits reproductive activity to one or a few individuals. Ovarian activity is inhibited in the majority of nestmates, including those individuals that have already eclosed into a winged reproductive adult form, the alates. It is the alates that will eventually disperse away from their natal nest to initiate new colonies (Heath, 1903; Castle, 1934) as primary reproductives (Thorne, 1996), but these new queens must undergo a final stage of maturation before they can begin to produce eggs. This period of ovarian development is inhibited by stimuli from healthy functional queens (Castle, 1934; Greenberg & Stuart, 1979; Brent *et al.*, 2005) and from other female alates (Greenberg & Stuart, 1979; Brent & Traniello, 2001b) found within their natal nest. Until removed from these inhibitory signals, the ovaries of alates are inactive and contain relatively few functional ovarioles (Castle, 1934; Brent & Traniello, 2001a, b). After leaving their natal nest, alates quickly locate a potential nesting site, drop their wings, and excavate a brood chamber. During this period, these new queens usually pair with a male, but sometimes females fail to find a mate or end up nesting with multiple males or females (Heath, 1903). Disinhibition permits these new queens to begin the final stage of their development prior to vitellogenesis and oviposition, but their new social environment can still influence their maturation. Specifically, the rate of their development can be enhanced by stimuli from a reproductive male, but retarded by stimuli from another maturing female reproductive (Brent & Traniello, 2001b). For these social signals to have an impact on the queen's development, they would have to act through the endocrine system.

The inhibitory effect of functional queens on alate reproductive physiology probably results from manipulation of circulating concentrations of juvenile hormone (JH) and ecdysteroids (Brent *et al.*, 2005). These two hormones are the primary regulators of reproductive maturation and ovarian activity in the majority of insect species (Goodman & Granger, 2005; Raikhel *et al.*, 2005; Swever *et al.*, 2005). In termites, JH is involved in regulating caste differentiation (Lüscher, 1960, 1972, 1974, 1976; Yin & Gillot, 1975; Okot-Kotber, 1980, 1982, 1983; Nijhout & Wheeler, 1982; Greenberg & Tobe, 1985; Noirot & Bordereau, 1990; Okot-Kotber *et al.*, 1993) and there is evidence to suggest that JH induces vitellogenesis (Lüscher, 1960; Vieau & Lebrun, 1981; Scharf *et al.*, 2005). Circulating ecdysteroids can also be found in termite queens (Bordereau *et al.*, 1976; Delbeque et al., 1978; Brent *et al.*, 2005) and have been implicated in regulating the timing of egg production (Raina *et al.*, 2003; Brent *et al.*, 2005), possibly by influencing the synthesis, release or uptake of JH (Brent *et al.*, 2005). It is likely that modulation of one or both of these hormones is the means by which nestmates can influence the development of maturing queens. In the present study, this model of endocrine mediated developmental responses is tested by exposing maturing female alates to various influential social stimuli and monitoring subsequent changes in JH, ecdysteroids and ovarian status.

Here, the endocrine and developmental effects of manipulating the social environment of a maturing *Z. angusticollis* queen are investigated during the first 30 days of her maturation after disinhibition from signals associated with her natal nest. The study aims to determine whether the pace of reproductive maturation is set by hormonal responses to stimuli known to slow or accelerate development, and to assess the strength of the relationship between ecdysteroid titre and JH production during this period.

Materials and methods

Termites were chosen from stock colonies of *Z. angusticollis* collected from the Del Monte Forest in Pebble Beach, CA, in September 2003 and 2004, respectively. Parent colonies were kept in plastic boxes containing moistened nest wood maintained in an environmental chamber under an LD 14 : 10 h photoperiod at 23 °C. All termites used in the experiments were selected randomly from multiple colonies. Female alates attempting to disperse upon opening the parent colony were dewinged and placed in covered 67 mL plastic cups (Solo Cup Co., Highland Park, IL). The cups contained 2 g (dry weight) of birch sawdust moistened with distilled water. Individual females were allowed to mature for up to 30 days after removal from the natal nest, under one of three social conditions: (i) paired with a newly dewinged male reproductive; (ii) paired with another maturing female reproductive; or (iii) left in isolation.

The rate of JH production of paired corpora allata glands (CA) dissected from individual maturing primary females was measured *in vitro* using a rapid partition radiochemical assay (RCA) (Pratt & Tobe, 1974; Tobe & Pratt, 1974; modified by Feyereisen & Tobe, 1981) as described previously (Brent & Vargo, 2003; Brent *et al.*, 2005). JH III is the primary homologue of this hormone synthesized in *Z. angusticollis* (Meyer *et al.*, 1976; Greenberg & Tobe, 1985; Brent *et al.*, 2005). Maturing females were destructively sampled after being allowed to mature in a particular social context after 5, 10, 15 and 30 days. At least 16 females were sampled for each day. Additional samples were obtained from 23 virgin queens prior to isolation (day 0). The paired CA glands were dissected from immobilized termites, cleaned of attached tissue, and preincubated for 30 min in a Petri-dish at 26 °C in 100 μL of modified TC199 medium (Specialty Media, Phillipsburg, NJ), with 50 mm Hepes buffer, pH 7.4, without methionine or bicarbonate (to deplete intraglandular methionine), and containing 2% Ficoll 400 (Sigma Chemical Co., St Louis, MO). After preincubation, the CA were transferred to a 6 × 50 mm borosilicate culture tube containing 100 μL fresh medium supplemented with L-[methyl-^{3}H]-methionine (specific activity of 2.7–3.0 TBq mmol^{-1}; PerkinElmer Life Sciences, Inc., Boston, MA) at a final concentration of 1.85 kBq μL^{-1}. Culture tubes were maintained at 26 °C and were rotated at 90 r.p.m. at a 15° pitch on an orbital shaker for 5 h. Under these conditions, the rate of incorporation of L-[methyl-^{3}H]-methionine into JH III is constant for at least 6 h (Brent *et al.*, 2005). After incubation, radiolabelled JH was extracted from the medium and CA together using 250 μL of ice-cold iso-octane. A 100-μL aliquot from each sample was evaporated under N_2, and then mixed with 3 mL Scintiverse BD (Fisher, Pittsburgh, PA) scintillation fluid. Radiolabelled methionine incorporation was measured using a scintillation counter (Beckman LS-5801; Beckman Coulter, Inc., Fullerton, CA).

Ecdysteroid titre was determined using a modified radioimmunoassay developed by Warren *et al.* (1984) and Zera & Bottsford (2001), as described previously (Brent *et al.*, 2005). Using a glass micropipette, haemolymph was collected from three to seven maturing queens at the same stage of development. The haemolymph was pooled for a final volume of 10 μL and extracted with 500 μL of chilled 90% methanol. Samples were lyophilized, resuspended in 1 mL of methanol, and stored at –80 °C until analysed. Duplicate 10-μL aliquots of each sample were incubated overnight with 100 μL of [^{3}H]-ecdysone (specific activity of 1.9–4.1 TBq mmol^{-1}; PerkinElmer Life Sciences, Inc.) in Borate Buffer, and 100 μL of a polyclonal ecdysteroid antiserum (H-22 antibody, gift from L. Gilbert) at 4 °C on an orbital shaker. The antiserum has been shown to be cross-reactive for ecdysone, ecdysterone, 20-hydroxyecdysone and makisterone A (Warren & Gilbert, 1986). A standard competition curve was generated using 20-hydroxyecdysone (Sigma) in quantities from 15.6–2000 pg. After 18 h, 20 μL of cleaned Protein-A solution (Pansorbin; CalBiochem, San Diego, CA) was added to each tube to precipitate the complex during another 1 h of incubation at room temperature. Samples were centrifuged at 5000 *g* and the remaining pellet was washed twice with 100 μL borate buffer. Radioactivity was determined by a scintillation counter and ecdysteroid concentrations were estimated by nonlinear regression.

Ovarian development was assessed for days 0 and 30 by dissecting the queens in 70% ethanol under a dissecting microscope and counting the total number of functional ovarioles and the number of vitellogenic terminal oocytes as described in Brent & Traniello (2001a). Ovarioles were considered functional if they were not filamentous and contained oocytes at some stage of development. Oocytes were considered vitellogenic if yolk protein could be observed and with volume $\geq$ 0.01 mm^3 (Hewitt *et al.*, 1972; Brent & Traniello, 2001b).

Hormone data were analysed for differences among treatment groups by analysis of variance (ANOVA) with results adjusted by the Holm–Šidák method to reduce experiment-wise error rate for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

The rate of JH production varied significantly among females exposed to different social stimuli (Fig. 1a). Prior to removal from their natal nest, CA from alates produce JH at a relatively high rate (3.57 ± 0.84 pmol h^{-1}, $n = 34$). After being paired with a male, CA from maturing queens began to produce significantly less JH after 5 days (0.85 ± 0.29 pmol h^{-1}, $n = 23$; ANOVA, $t = 4.284$, $P < 0.05$), and the rate remained low through day 30 (0.95 ± 0.47 pmol h^{-1}, $n = 22$), as was shown previously (Brent *et al.*, 2005). JH production in CA from females paired with another female did not change significantly between days 0 and 15 (2.50 ± 0.88 pmol h^{-1}, $n = 16$; ANOVA, $t = 1.459$, $P > 0.05$), but declined significantly by day 30 (0.27 ± 0.09 pmol h^{-1}, $n = 17$; ANOVA, $t = 4.612$, $P < 0.05$) and became equivalent to that for CA from females paired with a male (ANOVA, $t = 4.612$, $P < 0.05$). JH production in CA from queens left to mature in isolation began to decline after 5 days (1.89 ± 0.73 pmol h^{-1}, $n < 16$), but did not significantly differ from the rate observed in glands from day 0 females until day 10 (0.28 ± 0.08 pmol h^{-1}, $n = 18$;

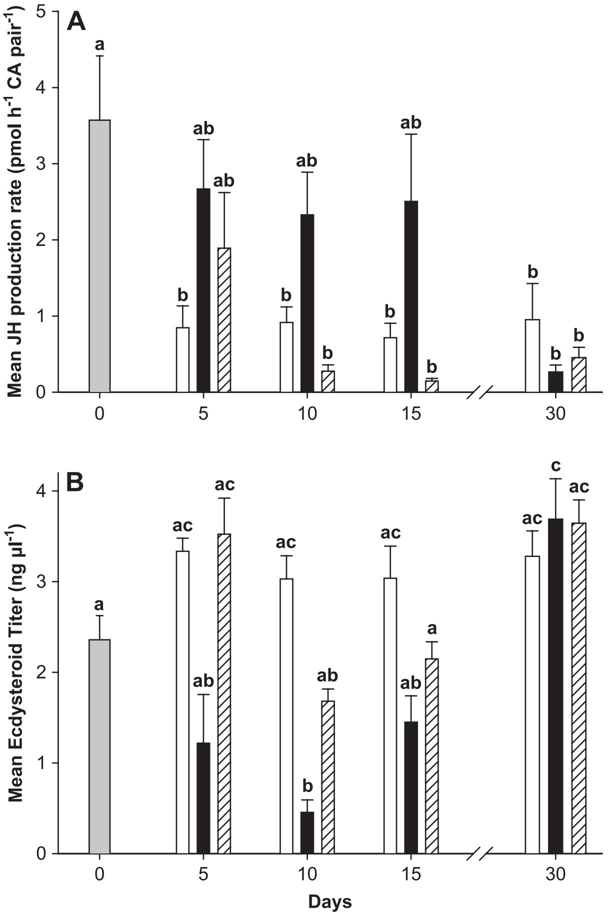

Fig. 1. Rates of juvenile hormone (JH) production (mean ± SE) *in vitro* (A) and ecdysteroid titres (B) in reproductive females sampled directly from their natal nest on day 0 (grey bars), and in maturing queens nesting with a reproductive male (open bars), with another maturing female (closed bars) or in isolation (hatched bars). Each mean represents 16–23 measures of JH production and 4–13 measures of ecdysteroid titre. Bars with the same letter are not significantly different ($P < 0.05$) by one-way ANOVA adjusted by the Holm–Šidák method.

ANOVA, $t = 4.686$, $P < 0.05$). The rate stayed low through day 30 (0.45 ± 0.14 pmol h^{-1}, $n = 21$).

Ecdysteroid titres, like JH production, varied over the sample period and between treatment groups (Fig. 1b). Females sampled directly from their natal nest on day 0 had a moderate titre of ecdysteroids in their haemolymph (2.36 ± 0.27 ng μL^{-1}, $n = 11$). The titres of females paired with a male did not differ significantly from this starting value on any single sample day; however, the average titre across the 5–30-day period was significantly elevated (3.14 ± 0.14 ng μL^{-1}, $n = 22$; ANOVA, $t = 2.581$, $P < 0.05$) relative to the day 0 ecdysteroid concentration. Titres in females exposed to stimuli from another female declined initially, reaching a low titre of 0.415 ± 0.17 ng μL^{-1} by day 10 ($n = 4$; ANOVA, $t = 4.388$, $P < 0.05$), but then rose over succeeding days until on day 30 the titre was comparable with that found in females reared with a male (3.69 ± 0.45 ng μL^{-1}, $n = 6$; ANOVA, $t = 0.970$, $P > 0.05$). Titres for females maturing in isolation varied over the period sampled, but never deviated significantly from that found in day 0 females. Only between days 5 and 10, when the titre declines by 52% from 3.52 ± 0.40 ng μL^{-1} ($n = 6$) to 1.68 ± 0.13 ng μL^{-1} ($n = 6$), was a significant change observed (ANOVA, $t = 4.211$, $P < 0.05$). On day 30, the titre for the isolated females was equivalent to that for females maturing with a male (3.65 ± 0.26 ng μL^{-1}, $n = 5$; ANOVA, $t = 0.821$, $P > 0.05$).

The relationship between JH production and ecdysteroid titre varied between females exposed to different social environments. A significant negative correlation was found for females nesting with a male (linear regression, $r = -0.92$, $F = 17.090$, $P < 0.05$), consistent with previous findings (Brent *et al.*, 2005). There was no significant relationship between the two hormones, however, for females nesting with another female ($r = -0.92$, $F = 1.457$, $P > 0.05$) or in isolation ($r = 0.12$, $F = 0.040$, $P > 0.05$).

Commensurate with the observed hormonal differences, females maturing under different social conditions exhibited varying rates of ovarian development during the 30 day study (Fig. 2). The mean number of functional ovarioles, when compared with females sampled on day 0 (30.31 ± 0.92, $n = 21$), increased in those nesting with a male (32.46 ± 0.75, $n = 21$), decreased in females nesting with another female (28.36 ± 0.99, $n = 18$), and did not change in females left in isolation (30.61 ± 1.03, $n = 21$). Similarly, the number of vitellogenic oocytes, when compared with those in day 0 females (4.00 ± 0.43, $n = 21$), increased in females nesting with a male (4.71 ± 0.54, $n = 21$), but decreased for females maturing with another female (2.76 ± 0.54, $n = 18$) or in isolation (2.94 ± 0.64, $n = 21$). The difference, after 30 days, between females nesting with either a male or with a female was significant for both ovariole number (ANOVA, $t = 3.211$, $P < 0.05$) and oocyte number (ANOVA, $t = 2.480$, $P < 0.05$).

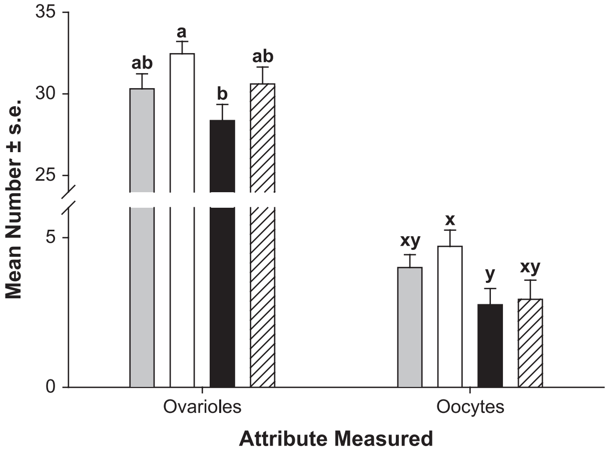

Fig. 2. The number of functional ovarioles and vitellogenic oocytes (mean ± SE) found in the ovaries of reproductive females sampled directly from their natal nest on day 0 (grey bars), and after maturing for 30 days with either a reproductive male (open bars), another maturing female (closed bars) or in isolation (hatched bars). Each mean represents 18–21 determinations. Bars with the same letter are not significantly different ($P < 0.05$) by one-way ANOVA adjusted by the Holm–Šidák method.

Discussion

In termites, it is not until a female alate is removed from the inhibitory stimuli encountered within her natal nest that substantial ovarian development and oocyte production occurs. Although separation from a functional queen appears to be the primary releaser cue for ovarian maturation and activity in alates of *Z. angusticollis* (Castle, 1934; Greenberg & Stuart, 1979; Brent *et al.*, 2005), an alate's maturation is still influenced strongly by the social environment to which she is subsequently exposed (Light & Illg, 1945; Light & Weesner, 1951; Greenberg & Stuart, 1979; Brent & Traniello, 2001a, b). Females nesting with a male tend to develop faster than those nesting with another maturing female or those left in isolation (present study; Brent & Traniello, 2001b). The results of the present study suggest that different social conditions expose maturing females to environmental signals that pace the endocrine system and result in variable rates of ovarian development.

JH has gonadotropic effects in the majority of insect species (Raikhel *et al.*, 2005), and current evidence indicates a similar function in *Z. angusticollis* (Greenberg & Tobe, 1985; Brent *et al.*, 2005). The hormone appears to perform a dual role in termite queens (Brent *et al.*, 2005); an elevated JH titre in immature alates may maintain reproductive inhibition, but an elevated JH titre in mature queens may stimulate ovarian activity. The present experimental design examined only the transition from reproductive inhibition, when JH production and JH titre are moderate, to an early phase of ovarian activity, when JH production and JH titre begin to rise (Brent *et al.*, 2005).

A significant decline in JH production is observed in all maturing queens, but its timing varies with the social conditions to which they are exposed (Fig. 1a). Functional queens appear to inhibit ovarian activity in alates by stimulating an elevated rate of JH production, preventing the final stages of reproductive maturation (Brent *et al.*, 2005). The significant decline in JH production after disinhibition observed in the alates of this experiment is probably a direct result of a cessation of the inhibitory effect. Stimuli from various nestmates appear to modify the pacing of this endocrine response, with a decline in JH production observable by day 5 for CA from females nesting with a male, by day 10 for glands from females nesting in isolation, and by day 30 for those from females nesting with another maturing queen. The timing of the decline in the synthesis of JH corresponds well with differential rates of ovarian development (Fig. 2). If it is assumed that females developing in isolation represent a condition of simple social disinhibition, then it appears that male-specific stimuli speed the rate of ovarian development by accelerating the rate of hormonal changes, whereas female-specific stimuli prolong the developmental inhibition by maintaining an elevated rate of JH production.

Ecdysteroid titres also change with time and vary according to the social stimulus provided, but the responses to the social conditions are somewhat less predictable than the changes in JH production. In most insects, ecdysteroids can direct reproductive development by regulating gene expression (Raikhel *et al.*, 2005). The role that ecdysteroids play during early stages of termite reproductive development is unknown but, in conjunction with JH, it is probable that they regulate organizational changes in the reproductive physiology of maturing queens (Noirot & Bordereau, 1990). Circulating ecdysteroids might also have a modulatory effect on JH production, specifically by restraining CA activity. In the closely-related cockroach *Diploptera*, ecdysteroids suppress CA activity (Stay *et al.*, 1980; Lanzrein *et al.*, 1981; Rankin & Stay, 1987; Chiang *et al.*, 1995) by promoting the release of allatostatins from the corpora cardiaca and/or by causing the CA to become more responsive to these inhibitory neuropeptides (Tobe & Stay, 1985; Stay *et al.*, 1991; Stay & Fairbairn, 1996, Stay *et al.*, 1997; Goodman & Granger, 2005). Allatostatin-immunoreactivity is found in axons within the CA of all life stages of the termite *Reticulitermes flavipes*, and cockroach allatostatin inhibits the CA of secondary reproductives (Yagi *et al.*, 2005), suggesting that cockroaches and termites may share similar endocrine regulatory mechanisms. There is some evidence in the present study supporting such an inverse relationship in *Zootermopsis*, with JH production falling as the ecdysteroid titre rises in females paired with males. However, this negative relationship is much weaker in females maturing with another female or in isolation. Large fluctuations between sample days could occur in one hormone without appreciable changes in the other, suggesting that although both hormone systems respond to the social stimuli, their responses are not directly coupled. More research is needed to understand fully the relationship between these two hormones, and the role that ecdysteroids play in termite reproductive development.

The evolution of a maturing queen's responsiveness to social stimuli from male and female reproductives is probably the result of selective pressures at different parts of a queen's life cycle. The enhanced rate of development observed in females nesting with a male is probably selected for during this incipient stage of colony development. The more rapidly a queen can rear her first brood, the greater her chance of successfully founding a new colony (Oster & Wilson, 1978; Shellman-Reeve, 1997). However, the best strategy for a queen might be to wait to invest her limited endogenous resources until a reproductive male becomes available to provide much-needed assistance with colony tasks (Shellman-Reeve, 1990, 1994, 1997; Rosengaus & Traniello, 1991; Brent & Traniello, 2001a, 2002) and sperm to fertilize her eggs. Although *Z. angusticollis* is one of several termite species capable of reproducing parthenogenically (Light, 1944), this is probably a contingency strategy, so that if a mate does not become available within a critical window of time after a queen has left her natal nest, she could still enhance her fitness by attempting to raise offspring by herself; females would have a much greater chance of success if a mate were available to provide support (Shellman-Reeve, 1990, 1994, 1997). This might explain why females maturing in isolation experience delayed but not inhibited ovarian development.

The inhibitory stimuli produced by another mature female is likely to have been selected for during the nonreproductive alate stage, because females do not usually nest together for long, if at all, after leaving their natal nest (Shellman-Reeve, 1994; but see also Heath, 1903). Large numbers of same sex alates housed together, even when a functional queen is not present, will not mature and thus appear to have an inhibitory effect on each other (Greenberg & Stuart, 1979). This mutual suppression may have evolved to hold all alates at the same developmental state, regardless of differences in the timing of their adult moult. Being at similar states of preparedness would facilitate coordinated swarming of groups of alates away from the natal nest, reducing the chances that any one individual might be targeted by predators. The inhibitory stimulus produced by alates may be as simple as a caste-specific recognition cue, such as an associated odour or behaviour (Stuart, 1979; Keller & Nonacs, 1993), or it may be a primer pheromone that can directly manipulate the endocrine system (Miller, 1969; Lüscher, 1974, 1976; Bordereau, 1985). The stimulus could also derive from the same inhibitory signal that functional queens produce. However, on an individual basis, an alate's suppressive influence on nestmates is much less than that of a functional queen's, given that paired females only experience delayed maturation rather than a complete inhibition. It is only in larger groups of alates that full inhibition can take place (Greenberg & Stuart, 1979). At some point during the period of development subsequent to disinhibition, it is likely that maturing queens stop producing the inhibitory stimuli of an alate, and begin to produce the stronger inhibitory stimuli of a functional queen. Alternatively, this system may involve quantitative modulation of the same signal, with functional queens producing much more of the signal than maturing alates.

The specific nature of the social stimuli to which maturing queens are responding is not yet known. The experimental design used in the present study does not separate physiological from behavioural signals, so that queens maturing with nestmates could be reacting to a variety of pheromonal, tactile, and visual stimuli. Females in heterosexual pairs may also receive physiological signals from copulation, such as physical and/or chemical factors that could modulate their endocrine physiology and behaviour (Gillott, 2003). Previous termite studies have attempted to isolate and identify the relevant stimuli (Castle, 1934; Lüscher, 1960, 1972; Greenberg & Stuart, 1979), but these used relatively insensitive measures of gross development over periods of weeks or months to monitor for signal activity in these slowly maturing insects. Most of those experiments have produced ambiguous and inconclusive results (Stuart, 1979). The results of the present study highlight the relative rapidity of the hormonal responses to exogenous stimuli and, hopefully, facilitate the design of new bioassays to determine the nature, composition, and origin of these developmentally influential cues.

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