

Effects of the NMDA receptor antagonist MK-801 on female reproduction and juvenile hormone biosynthesis in the cricket *Gryllus bimaculatus* and the butterfly *Bicyclus anynana*

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SUMMARY

Apart from regulating insect development, juvenile hormones (JHs) play an important role in insect reproduction, where they initiate vitellogenin synthesis and regulate the uptake of yolk by the ovary. JH synthesis is a tightly regulated process controlled by neurons and peptidergic neurosecretory cells. One of the known stimulatory regulators of JH biosynthesis is glutamate, and its *N*-methyl-D-aspartate (NMDA) receptor has been recently found in the cockroach *Diploptera punctata*. In this study we demonstrate a strong reduction in reproductive output in the tropical butterfly *Bicyclus anynana* and the Mediterranean field cricket *Gryllus bimaculatus* caused by the NMDA receptor antagonist MK-801. Such inhibiting effects on reproduction could be overruled by the application of JH mimics. In *G. bimaculatus*, MK-801 inhibits *in vitro* JH biosynthesis in the corpora allata and reduces *in vivo* JH haemolymph titres in a dose-dependent manner. These results suggest that JH biosynthesis in the corpora allata is at least in part controlled by an NMDA receptor with Ca²⁺ as a second level messenger. Based on our findings we consider NMDA receptor antagonists as important tools for manipulating juvenile hormone biosynthesis and therefore for gaining a better understanding of the mechanistic basis of reproduction.

Key words: fecundity, *Corpora allata*, glutamate receptor, insects.

INTRODUCTION

In insects, hormones are the main regulators of larval development, metamorphosis, behaviour, caste determination, diapause, polymorphism and reproduction (Flatt et al., 2005; Gäde et al., 1997; Nijhout, 1994). Among the different hormones, the juvenile hormones (JHs) and the ecdysteroids are generally considered the most important ones in affecting these processes in insects. JHs are sesquiterpenoid lipid-like compounds secreted by the corpora allata (CA), a pair of epithelial glands, and are well-known for preventing metamorphosis in the insect juvenile stages and for having pronounced effects on reproduction in the adults (Gilbert et al., 2000; Nijhout, 1994; Ramaswamy et al., 1997). Their primary function in reproduction is to initiate vitellogenin synthesis in the fat body and to regulate the uptake of yolk by the ovary (Hoffmann, 1995). JH biosynthesis is a tightly regulated process involving stimulating (allatotropins) and inhibiting (allatostatins) neuropeptides secreted by brain neurosecretory cells (Kataoka et al., 1989; Stay and Tobe, 2007), and also classical neurotransmitters (Chiang et al., 2002a; Granger et al., 2000; Liu et al., 2005; Rachinsky and Tobe, 1996).

At least in the cockroach *Diploptera punctata*, CA function is controlled by either stimulating ionotropic (Chiang et al., 2002a) or inhibiting metabotropic receptors (Liu et al., 2005). The identified ionotropic *N*-methyl-D-aspartate (NMDA) glutamate-gated receptor in the CA of *D. punctata* has striking similarities with the vertebrate NMDA receptor, especially regarding its structure, high Ca²⁺ permeability, and its response to typical antagonists such as MK-801, conantokin and Mg²⁺ (Chiang et al., 2002a). Interestingly, NMDA receptors in mammals are important for reproductive

control through their effects on the release of the gonadotropin-releasing hormone required for the initiation of puberty, the maintenance of reproductive capability and reproductive behaviour (Mahesh and Brann, 2005). Similar effects have been described in other vertebrates (Flynn et al., 2002), and also in a protochordate species, *Ciona intestinalis*, suggesting a highly conserved reproductive function in chordates (D'Aniello et al., 2003; Di Fiore et al., 2000).

MK-801, as a high-affinity antagonist of NMDA receptors (Wong et al., 1986), has been intensively studied in mammalian models, in particular in connection with its effects on neuronal plasticity and its neurotoxicity-reducing effects in ischaemia, epilepsy, brain hypoxia and hypoglycemia (Ellison, 1995; Williams et al., 1991; Wolf, 1998). Furthermore, this antagonist was used to study NMDA receptor-mediated effects on reproduction in other vertebrates (Flynn et al., 2002; Luderer et al., 1993; Mahesh and Brann, 2005; Melis et al., 2004). By contrast, in insects MK-801 has thus far only been used in three species. MK-801 was found to inhibit NMDA-triggered JH biosynthesis *in vitro* in the cockroach *D. punctata*, by reducing levels of free cytosolic calcium in the CA (Chiang et al., 2002a; Chiang et al., 2002b). Furthermore, Begum and co-workers (Begum et al., 2004) used MK-801 as an efficient blocker of vitellogenesis in the flesh fly *Neobellieria bullata* and the locust *Schistocerca gregaria*. However, studies on long-term effects of MK-801 on reproduction and on JH biosynthesis, involving *in vitro* and *in vivo* measurements, are apparently missing (Begum et al., 2004). We address these issues here using two insect species: the hemimetabolous cricket *Gryllus bimaculatus* and the holometabolous butterfly *Bicyclus anynana*, in order to validate the

above supposed mode of action and to test for its generality (Zera, 2007).

Gryllus bimaculatus has been extensively used to study the hormonal control of reproduction, which strongly depends on JH (Hoffmann et al., 1996; Lorenz, 2003; Lorenz et al., 1995a; Lorenz et al., 1995b), thus making it a highly suitable target for JH manipulation. The butterfly *B. anynana* belongs to a group of the Lepidoptera in which vitellogenesis and choriogenesis seem to depend exclusively on JH (Ramaswamy et al., 1997). Reproduction, including different strategies in response to prevailing temperatures, has been extensively studied in *B. anynana* (Fischer et al., 2003a; Fischer et al., 2004; Fischer et al., 2003b; Steigenga et al., 2005), but its hormonal control is hitherto only poorly understood (Steigenga et al., 2006), making this species another suitable model. Given the dependence of reproduction on JH in both species, we here examine the effect of the NMDA receptor antagonist MK-801 on lifetime fecundity and egg size, on *in vitro* and *in vivo* JH biosynthesis, and its interactions with JH mimics.

MATERIALS AND METHODS

Animals and experimental populations

For this study two species of insects, the tropical butterfly *Bicyclus anynana* Butler 1879 (Lepidoptera, Satyriinae) and the Mediterranean field cricket *Gryllus bimaculatus* de Geer 1773 (Ensifera, Gryllidae) were used. *B. anynana* is a fruit-feeding butterfly with a distribution ranging from Southern Africa to Ethiopia (Larsen, 1991). A laboratory stock population was established at Bayreuth University, Germany, in 2003 from several hundred individuals derived from a well-established stock population at Leiden University, The Netherlands. The Leiden population was founded in 1988 from over 80 gravid females caught at a single locality in Malawi. Several hundred adults are reared in each generation, maintaining high levels of heterozygosity at neutral loci (Van't Hof et al., 2005). *G. bimaculatus* has a global distribution, spanning Africa, Asia and southern Europe (Harrison and Bogdanowicz, 1995; Ragge, 1972). The laboratory colony at Bayreuth University was established with field-caught animals from Italy and Spain in 1995. Regularly, individuals (also originating from Mediterranean areas) from commercial suppliers were added to the stock population to maintain high levels of heterozygosity (Lorenz et al., 2004).

Insect rearing

Bicyclus anynana was reared in a climate cell at 27°C, 70% relative humidity, and a photoperiod of 12 h:12 h light:dark. Larvae were fed on young maize plants in population cages (50 cm×50 cm×80 cm). The resulting pupae were collected from the plants and transferred to cylindrical hanging cages. Throughout all experiments, butterflies had access to moist banana for adult feeding. *G. bimaculatus* was reared at 27°C, 30–40% relative humidity and a photoperiod of 16 h:8 h light:dark. Larvae were reared in population cages (45 cm×40 cm×65 cm), fed on a mixture of commercial rat/mouse, rabbit and cat diets (Altromin GmbH, Lage, Germany), and supplied with drinking water *ad libitum*. Newly ecdysed adults (day 0) were collected daily and transferred to population cages (22 cm×20 cm×37 cm).

Experimental design

To investigate the effects of the NMDA receptor antagonist MK-801 on female reproduction and juvenile hormone biosynthesis, five different experiments were performed as outlined below.

Experiment 1: effects of MK-801 on *B. anynana* reproduction

On the day of eclosion, female butterflies were randomly divided among four treatment groups, being treated with 0 (control), 10, 20 or 30 µg MK-801 in 4 µl Ringer solution. These solutions were repeatedly injected into the females' thorax, using a Hamilton syringe, on days 0, 2 and 6 of adult life. All females were kept together with male butterflies for mating until day 2 of adult life. After the mating period, females were placed individually in translucent plastic containers (1 l, covered with gauze) containing a fresh cutting of maize for egg-laying. Eggs of ~40 females per group were collected and counted daily until the death of the females. Egg size was measured as cross-sectional area (mm²) using a digital camera (Leica DC300, Leica Microsystems, Wetzlar, Germany) connected to a stereo microscope (Leica MZ 7.5). The resulting images were analysed using Scion Image public software (Scion Corporation 2000, Frederick, Maryland, USA). Tight correlations between egg area (applying image analysis) and egg mass as well as hatchling size confirm that this method provides a highly reliable measurement of egg size in *B. anynana* (Fischer et al., 2002).

Experiment 2: effects of MK-801 on *G. bimaculatus* reproduction

Adult female crickets were randomly divided among three treatment groups, being injected with 0 (control), 50, or 150 µg MK-801 in 4 µl DMSO–Ringer (1:1 v/v) solution (note that MK-801 is not soluble in pure Ringer at high concentrations). This solution was injected into the lateral intersegmental membrane between the third and fourth abdominal segment, using a Hamilton syringe, on days 0 and 3 of adult life. Females were housed together with males for mating from day 2 until day 4 following ecdysis. Thereafter, females were placed individually in plastic boxes (18×13.5×6 cm) and provided moist sand as egg laying substrate. Eggs were collected, counted and measured (as outlined above) daily for the following 8 days. For each group about 35 females were used.

Experiment 3: interactive effects between MK-801 and JH mimics in *B. anynana* and *G. bimaculatus*

To investigate whether any potential effects of MK-801 on reproductive traits are mediated through variation in JH titres, JH mimics were applied to artificially increase JH active compounds in the haemolymph. As mimics, pyriproxyfen (Dr. Ehrenstorfer GmbH, Augsburg, Germany) was used for *B. anynana*, and methoprene (Fluka, Taufkirchen, Germany) for *G. bimaculatus*. The compounds are known to work well as JH mimics in these species (Hoffmann et al., 1996; Steigenga et al., 2006). Both species were randomly divided among four treatment groups, being treated with MK-801, a JH mimic, MK-801 plus JH mimic or the pure solvent (control). *B. anynana* females (70–77 per group) were treated on days 0 and 2 with either 10 µg MK-801 in 6 µl Ringer (injected), 0.1 µg pyriproxyfen in 2 µl acetone (applied topically on the abdomen using a Hamilton syringe), both compounds or 6 µl Ringer–2 µl acetone (control). Females were kept together with males for mating until day 2, and were afterwards placed individually in plastic containers as described above. Egg numbers were determined on day 3 of adult life only. *G. bimaculatus* females (33–36 per group) were treated on days 0 and 3 with either 150 µg MK-801 in 4 µl DMSO–Ringer (1:1, v/v), 30 µg methoprene in 4 µl isooctane (applied topically on to the abdomen), both compounds or 4 µl DMSO–Ringer/4 µl isooctane (control). Females were kept together with males for mating until day 3 and were then placed individually in plastic containers as described above. Egg numbers were determined for days 4–6

following ecdysis. The respective concentrations and treatment days were chosen on the basis of experiments 1 and 2 as well as pilot studies.

Experiment 4: effects of MK-801 on *in vitro* JH biosynthesis in *G. bimaculatus*

Owing to the small size of the CA and very small amounts of haemolymph in *B. anynana*, experiments 4 and 5 were restricted to *G. bimaculatus*. Single CA from *G. bimaculatus* females were used in a rapid partition assay (Feyereisen and Tobe, 1981). Methods essentially followed those of Lorenz et al. (Lorenz et al., 1995b; Lorenz et al., 1997) with some modifications: the TC 199 incubation medium (M 7653, Sigma, Deisenhofen, Germany) with Hank's salts and sodium bicarbonate, without L-glutamine, buffered with 25 mmol l⁻¹ Hepes, supplemented with CaCl₂ to a final concentration of 3 mmol l⁻¹, L-methionine to a final concentration of 0.28 mmol l⁻¹ and sodium acetate to a final concentration of 2.5 mmol l⁻¹, fortified with 1% Ficoll 400, was adjusted to pH 7.2. As radiolabelled precursor, [¹⁴C₂]acetate (MC 213; Hartmann Analytic, Braunschweig, Germany) was added to a final specific activity of 64 MBq mmol⁻¹. The resulting total acetate concentration in the radiolabelled incubation medium was 2.58 mmol l⁻¹. Single glands without MK-801 were pre-incubated for 90 min to stabilize JH synthesis in the *in vitro* setup, then transferred to the first incubation for 120 min and finally assigned to the second incubation with the respective treatments for 120 min. JH release was examined in untreated control animals and at 6 MK-801 concentrations ranging from 10⁻³ to 10⁻⁶ mol l⁻¹. JH release rates are given relative to the initial incubation, to correct for differences between single CA (*N*=20–35, but for 10⁻³ and 10⁻⁶ mol l⁻¹, *N*=10).

Experiment 5: effects of MK-801 on *in vivo* JH titres in *G. bimaculatus*

The JH titres in the haemolymph of *G. bimaculatus* females were quantified by liquid chromatography–mass spectrometry (LC–MS) (Westerlund and Hoffmann, 2004). The experimental design followed the one described for experiment 2 with 21–24 females for each treatment. Three and 24 h after the second injection on day 3, 20 µl of haemolymph were collected per female and extracted (Westerlund and Hoffmann, 2004). The samples were separated on a C18 reverse-phased column (ReproSil-Pur ODS-3, 5 µm; Dr Maisch GmbH, Germany), protected by a guard column (C18 cartridge; Phenomex, Aschaffenburg, Germany) with differing gradients of water–methanol. MS analysis was accomplished using electrospray ionization (ESI) in positive ion mode using a Shimadzu LCMS-2010A. As only the relative differences between treatments were of importance, no additional calibration to estimate the absolute amount of juvenile hormone was applied.

Data analysis

Differences in egg numbers over time were analyzed using two-way repeated measurements ANOVAs, with treatment and time (i.e. oviposition day) as factors. Data on total fecundity, mean egg size (averaged over the oviposition period) and JH titres were analyzed with standard ANOVAs. As treatment with MK-801 frequently resulted in the production of zero eggs per day, no repeated measurements ANOVAs could be calculated for egg sizes. Differences among treatment groups were analysed using Tukey's HSD. The fecundity data from *G. bimaculatus* were square-root transformed prior to analyses to meet ANOVA requirements. Survival probabilities of *B. anynana* females over time were analyzed by survival analyses for multiple groups, based on Gehan's

generalized Wilcoxon test. The dose–response curve for the release of JH by the CA of *G. bimaculatus* with regard to MK-801 treatment was calculated using a sigmoidal 5-parameter fit of SigmaPlot 9.1. All statistical tests were performed using Statistica 6.1 and values are given as means ±1 s.e.m.

RESULTS

Experiment 1: effects of MK-801 on *B. anynana* reproduction

The number of eggs laid over time differed significantly across treatment groups (repeated measurements ANOVA: $F_{3,1370}=5.78$, $P<0.001$). Differences were particularly pronounced during the first days of the oviposition period (Fig. 1A). Following an initial increase, egg numbers generally declined with female age ($F_{10,1370}=53.17$, $P<0.001$). A significant difference in treatment over time (treatment by time interaction; $F_{30,1370}=5.31$, $P<0.001$) probably reflects the above mentioned pronounced differences in early fecundity, whereas egg numbers were more similar later in life. Note that the third injection of MK-801 on day 6 of adult life had only a minor effect on egg production (Fig. 1A). In line with the above results on daily fecundity, lifetime fecundity differed significantly across treatment groups, being reduced by ~24% in the 30 µg MK-801 group as compared to the control ($F_{3,156}=3.10$, $P=0.028$; Table 1A). Again, differences were most pronounced during the first days of the oviposition period ($F_{3,152}=10.43$, $P<0.001$; Table 1B).

Egg size generally decreased with increasing female age, but (if averaged over the whole oviposition period) did not differ

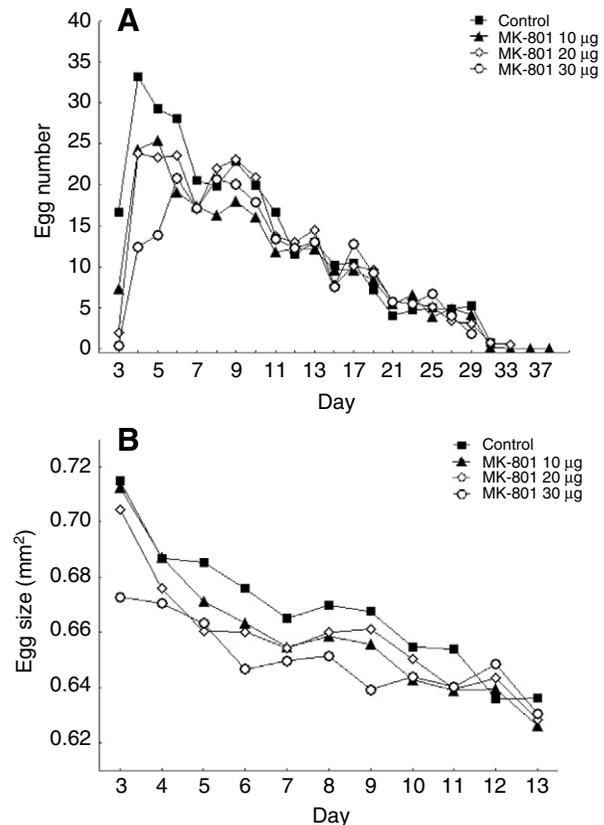


Fig. 1. Number (A) and size (B) of eggs produced over time by female *Bicyclus anynana* treated with different concentrations of MK-801. Injections of MK-801 (controls were injected with Ringer solution only) were given on days 0, 2 and 6 of adult life. To improve clarity, no standard errors are presented (*N*=38–40).

Table 1. Effects of MK-801 treatment on fecundity and mean egg size in *Bicyclus anynana* and *Gryllus bimaculatus*

	N	Fecundity		Mean egg size	
		Mean	s.e.m.	Mean	s.e.m.
(A) <i>Bicyclus anynana</i>					
Control	40	275.9	15.3	0.658	0.007
MK-801 10 µg	40	226.1	19.5	0.650	0.006
MK-801 20 µg	38	252.6	13.2	0.649	0.004
MK-801 30 µg	38	208.6	18.2	0.639	0.006
(B) <i>Bicyclus anynana</i>					
Control	40	126.5	7.8	0.684	0.007
MK-801 10 µg	40	91.1	6.6	0.675	0.006
MK-801 20 µg	38	89.6	6.8	0.667	0.005
MK-801 30 µg	38	59.4	6.5	0.656	0.006
(C) <i>Gryllus bimaculatus</i>					
Control	34	386.5	61.9	0.932	0.007
MK-801 50 µg	37	286.9	39.0	0.942	0.007
MK-801 150 µg	34	233.4	38.6	0.941	0.009

(A) Lifetime fecundity (i.e. total number of eggs produced throughout the life of a given female) and mean egg size for *B. anynana* (experiment 1); (B) egg numbers and mean egg size for days 3–7 after eclosion for *B. anynana* (experiment 1); (C) fecundity and mean egg size until day 12 after eclosion for *G. bimaculatus* (experiment 2).

significantly between treatment groups ($F_{3,155}=1.83$, $P=0.14$; Table 1A, Fig. 1B). However, restricting the analysis to days 3–7 of adult life shows that mean egg size tended to decrease with increasing MK-801 concentration ($F_{3,155}=4.00$, $P=0.009$; Table 1B). MK-801 treatment did not affect female survival probability ($\chi^2_4=2.5$, $P=0.47$; Fig. 2).

Experiment 2: effects of MK-801 on *G. bimaculatus* reproduction

Daily egg numbers differed significantly across treatment groups, being generally lower in the groups treated with MK-801 (repeated measurements ANOVA: $F_{2,642}=5.35$, $P=0.006$; treatment by time interaction $F_{12,642}=0.77$, $P=0.686$; Fig. 3A). Egg numbers peaked on day 6 of adult life, followed by a constant decline with female age ($F_{6,642}=67.79$, $P<0.001$). Accordingly, lifetime fecundity was significantly reduced (by ~40%) in the group treated with 150 µg

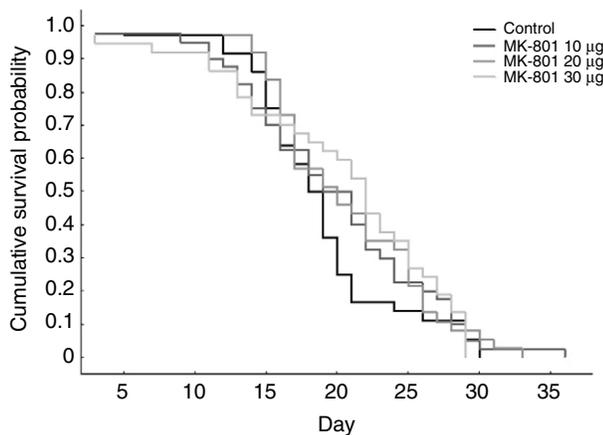


Fig. 2. Effects of MK-801 on the cumulative survival probability of *Bicyclus anynana* females. Injections of MK-801 (control: Ringer solution) were applied on days 0, 2 and 6 of adult life ($N=38-40$).

MK-801 as compared with the control group ($F_{2,104}=3.10$, $P=0.04$; Table 1C). Egg size was not significantly affected by MK-801 ($F_{2,103}=0.54$, $P=0.59$; Fig. 3B). As this experiment was terminated on day 12 following eclosion (coinciding with the end of egg laying), no longevity data are available, but at least during this phase mortality rates were very similar (control: 0 individuals; 50 µg MK-801: 2; 150 µg MK-801: 0).

Experiment 3: interactive effects between MK-801 and JH mimics in *B. anynana* and *G. bimaculatus*

Egg numbers varied significantly across treatment groups in both species (*B. anynana*: $F_{3,270}=9.99$, $P<0.001$; *G. bimaculatus*: $F_{3,139}=10.55$, $P<0.001$; Fig. 4A,B). They were reduced in the MK-801-treated groups, but increased in the groups treated with a JH mimic. Most interestingly, egg numbers were very similar to controls in the groups treated with both compounds.

Experiment 4: effects of MK-801 on *in vitro* JH biosynthesis in *G. bimaculatus*

JH synthesis in single *G. bimaculatus* CA decreased significantly with increasing amounts of MK-801 ($F_{5,135}=13.81$, $P<0.001$; Fig. 5). Maximal inhibition of ~57.4% occurred at the highest concentration (10^{-3} mol l⁻¹ MK-801), 50% inhibition was reached at about 1.5×10^{-4} mol l⁻¹. The control groups, untreated in the second

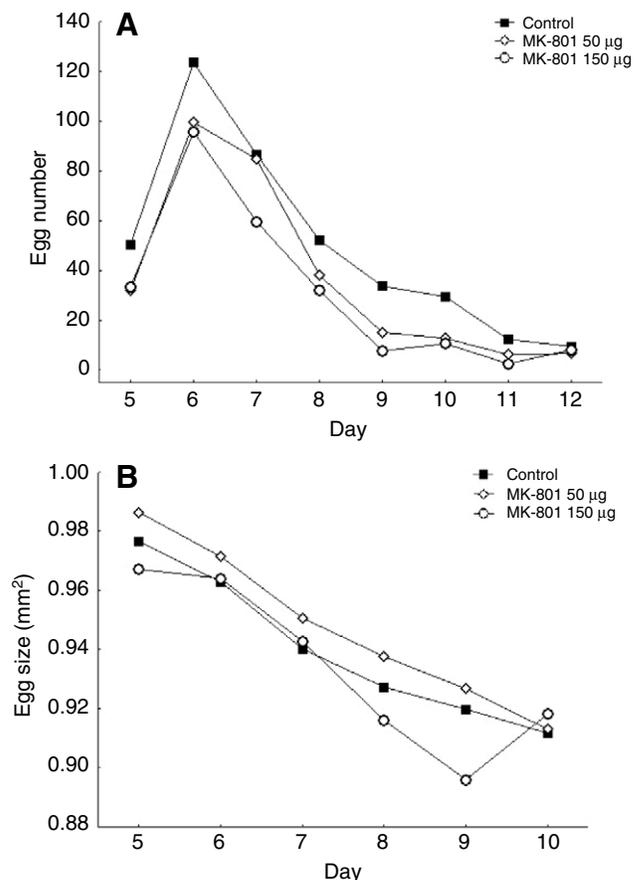


Fig. 3. Number (A) and size (B) of eggs produced over time by female *Gryllus bimaculatus* treated with different concentrations of MK-801. Injections of MK-801 (controls were injected with Ringer:DMSO 1:1, v/v) were given on days 0 and 3. To improve clarity, no standard errors are presented ($N=34-37$).

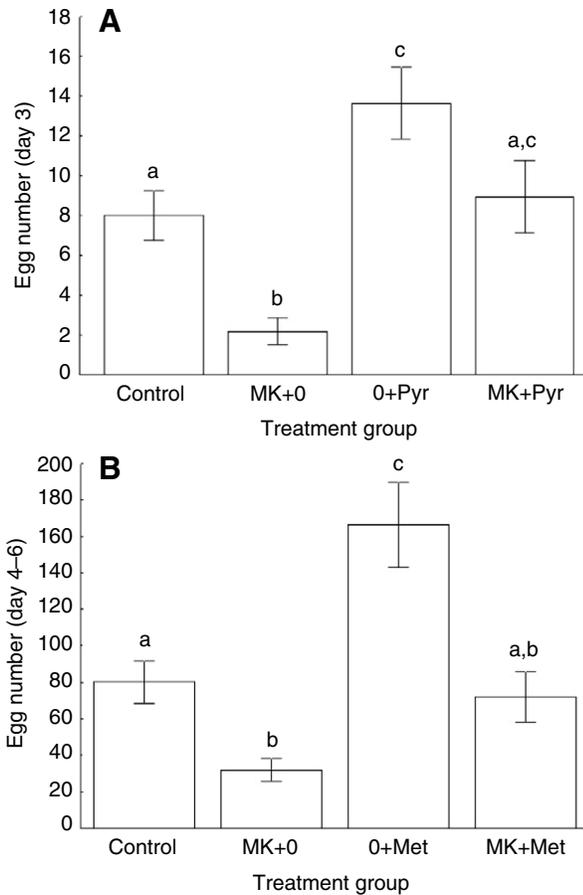


Fig. 4. Effects of MK-801 (MK), juvenile hormone mimics [pyriproxyfen (Pyr) or methoprene (Met)], both compounds or the pure solvent (control) on early fecundity in *Bicyclus anynana* (A; $N=70-77$) and *Gryllus bimaculatus* (B; $N=33-36$). Values are means ± 1 s.e.m. Different letters above bars indicate significant differences between groups (Tukey's HSD after ANOVA).

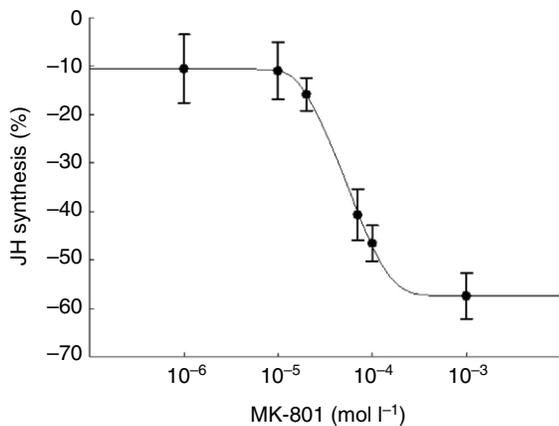


Fig. 5. Dose-response curve for *in vitro* inhibition of juvenile hormone (JH) synthesis by MK-801 in single *Gryllus bimaculatus* corporus allatum. Values are given relative to the first incubation rates of each corporus allatum ($N=20-35$, but for 10^{-3} and 10^{-6} mol l⁻¹ $N=10$). Controls released JH III in the second incubation at a rate of 26.8 ± 1.8 pmol h⁻¹.

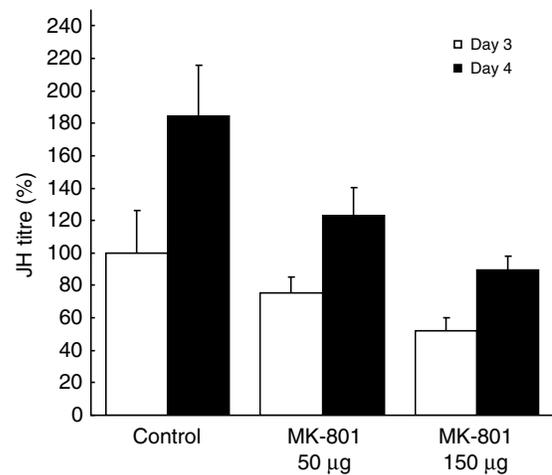


Fig. 6. Effects of MK-801 and time on *in vivo* juvenile hormone titres in the haemolymph of *Gryllus bimaculatus* females ($N=21-24$). Females were injected with MK-801 on days 0 and 3 following ecdysis. Haemolymph was sampled 3 and 24 h after the last injection. The value for the control group on day 3 was set to 100%. Given are means ± 1 s.e.m.

incubation, showed a JH synthesis of -1.9% compared with the first incubation, suggesting that JH synthesis remained stable over time (paired *t*-test: $t=1.86$, $N=42$, $P=0.13$).

Experiment 5: effects of MK-801 on JH titres *in vivo* in *G. bimaculatus*

Only JH III was detected in the haemolymph of *G. bimaculatus* females. Injection of MK-801 significantly decreased JH titres *in vivo*, maximally by 48.4% (repeated measurements ANOVA: $F_{2,65}=4.07$, $P=0.022$; Fig. 6). Furthermore, JH III titres increased significantly by $\sim 74\%$ from day 3 to 4 ($F_{1,65}=29.1$, $P<0.001$; treatment by time interaction $F_{2,65}=1.93$, $P=0.15$).

DISCUSSION

In both species studied, the NMDA receptor antagonist MK-801 clearly reduced reproductive output. Fecundity was reduced by up to 40% in *G. bimaculatus*, and by up to 24% in *B. anynana*. In the latter, egg size was additionally negatively affected by MK-801 (significantly so during the first days of oviposition), which was not the case in *G. bimaculatus*. For *B. anynana* detailed information on JH biosynthesis in the CA is lacking, but JHs generally play a key role in egg maturation in this group of lepidopterans (Ramaswamy et al., 1997). Furthermore, Steigenga et al. (Steigenga et al., 2006) demonstrated that applications of the JH mimic pyriproxyfen significantly increased fecundity but decreased longevity, supporting the notion that in *B. anynana* JH has pleiotropic effects on key life-history traits, as has been found for other insects (Flatt et al., 2005; Ramaswamy et al., 1997; Zera et al., 1998). For *G. bimaculatus* much more detailed information on JH biosynthesis and the hormonal control of reproduction, especially egg maturation, is available (Hoffmann et al., 1996; Koch and Hoffmann, 1985; Lorenz et al., 1997).

However, we propose that the overall similar reduction in reproductive output found in both species is causally related to the inhibitory effects of MK-801 on JH biosynthesis (Chiang et al., 2002a). Accordingly, we predicted that negative effects of MK-801 on fecundity can be restored by adding JH active compounds. Indeed

this was found when treating females with both, MK-801 and JH mimics, yielded fecundity data for both species that were statistically indistinguishable from those of the control groups. Similarly, Begum et al. (Begum et al., 2004) showed that JH treatment could overrule the blocking effect of MK-801 on vitellogenesis in *S. gregaria*. Although these findings strongly suggest that the NMDA receptor is involved in JH biosynthesis, a proof can only be obtained by *in vitro* and *in vivo* analyses (Begum et al., 2004; Zera, 2007).

Corresponding analyses in *G. bimaculatus* (performing the same measurements in *B. anynana* was not possible for practical reasons) showed a reduction of *in vitro* JH biosynthesis and *in vivo* haemolymph JH titres (by up to 48%) in MK-801-treated compared with control females. JH biosynthesis was inhibited successfully in *G. bimaculatus* CA by up to 60%, resembling the results of *in vitro* measurements of active CA glands in *D. punctata* (Chiang et al., 2002a). In the experiments of Chiang et al. (Chiang et al., 2002a) CA glands were incubated with NMDA to compensate for the missing glutamate stimulus from the severed nerves, with much lower concentrations of MK-801 needed for this degree of inhibition. The rise of JH III titres in the haemolymph between days 3 and 4 was expected, as in *G. bimaculatus* JH III titres reach their maximum shortly before the onset of egg laying (Koch and Hoffmann, 1985). Taken together, the available evidence leaves little doubt that MK-801 affects JH biosynthesis, and concomitantly JH titres, in both species.

The effects of MK-801 on JH biosynthesis in *G. bimaculatus* are possibly mediated through a glutamatergic NMDA receptor, acting on the Ca²⁺ flux and thereby on JH biosynthesis. In adults of the cockroach *D. punctata*, JH biosynthesis in the CA is initially sensitive to allatostatins but insensitive to ionotropic glutamate stimulation, resulting in low rates of JH synthesis. Mating changes this pattern towards insensitivity of the CA to allatostatins and a high response to glutamate stimulation, resulting in high rates of JH synthesis (Chang et al., 2005). *G. bimaculatus* allatostatins, by contrast, seem to act less age-dependently throughout the life cycle, although on days with maximum JH synthesis allatostatic inhibition is slightly lowered (Lorenz, 2001). However, calcium ions [with their influx being regulated by the NMDA receptor in *D. punctata* (Chiang et al., 2002a)], stimulate JH synthesis also in *G. bimaculatus* (Klein et al., 1993; Woodring and Hoffmann, 1994), and there is no interaction between allatostatins (or allatotropins) and Ca²⁺-mediated effects on JH biosynthesis (Lorenz, 2001). A further target of glutamate might also be a Na⁺-dependent transporter (Kosakai and Yoshino, 2001).

There is no indication of any toxic side effects of the compounds or solvents used that may have affected our results. For *B. anynana*, MK-801 was dissolved in Ringer solution, thereby minimizing any potential solvent effects. Indeed, lifetime fecundity in the control group was very similar to values obtained from other studies not involving injections or applications (Bauerfeind and Fischer, 2005; Bauerfeind et al., 2007). Furthermore, survival data revealed no difference among control and MK-801-treated groups, suggesting that MK-801 is a highly specific compound without any toxic side-effects. For *G. bimaculatus* it was necessary to use DMSO as solvent because of the much higher concentrations of MK-801 employed. Concomitantly, lifetime fecundity was generally lower than in other studies (Koch and Hoffmann, 1985; Lorenz, 2007; Meyering-Vos et al., 2006), but again, there was no detectable effect on mortality rates, although data were restricted to the egg laying period in this case.

Despite the overall similarity of effects in both species used, there were also some interesting differences in the effects of MK-801 on

fecundity. In *G. bimaculatus* egg production was reduced throughout the oviposition period (at least until day 11 following ecdysis), but in *B. anynana* the inhibitory effects of MK-801 were restricted to the first days of the oviposition period. Furthermore, the dose dependence of effects seems more pronounced in *B. anynana* than in *G. bimaculatus*. These findings may suggest some differences in the effects of JH on egg maturation across species. In *G. bimaculatus* JH biosynthesis and fecundity can be manipulated throughout the entire oviposition period by allatostatins and JH (mimic) injections administered early in life (Koch and Hoffmann, 1985; Lorenz, 2001). Therefore, egg maturation seems to depend on a constant input of JH-mediated signals in *G. bimaculatus*. In *B. anynana*, by contrast, JH seems to be an important signal for the initiation of egg maturation, which might not be needed later on (Steigenga et al., 2006).

In conclusion, the NMDA receptor antagonist MK-801 reduced fecundity in *G. bimaculatus* and *B. anynana*, two species not being phylogenetically closely related. This effect could be reversed by concurrent applications of JH mimics. Furthermore, MK-801 inhibited *in vitro* JH biosynthesis in the CA and reduced *in vivo* JH haemolymph titres in a dose-dependent manner in *G. bimaculatus*. These results suggest that in *G. bimaculatus* JH biosynthesis in the CA is at least in part controlled by an NMDA receptor with Ca²⁺ as a second level messenger, as has been found in the cockroach *D. punctata* (Chiang et al., 2002a). As MK-801 is readily available commercially, is fairly soluble in water and can be used orally, it obviously represents a convenient tool for manipulating JH biosynthesis in insects. With the growing knowledge on NMDA receptors in insects (Chiang et al., 2002a; Chiang et al., 2002b; Locatelli et al., 2005; Xia et al., 2005), such antagonists may yield new insights into the mechanistic basis of reproduction and associated trade-offs in insects.

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