Effects of the avermectin, MK-243, on ovary development and salivary gland degeneration in the ixodid tick, *Amblyomma hebraeum*

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Short Title: Physiological effects of avermectin MK-243 in ticks

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Abstract

Injection of the avermectin analogue, MK-243, into engorged female *Amblyomma hebraeum* Koch resulted in reduced ovary weight, oocyte length, and ovary vitellin content. There was no significant reduction in hemolymph vitellogenin concentration in MK-243 treated ticks. Although MK-243 was previously shown to markedly reduce hemolymph 20E-concentration, injection of 20E, the vitellogenic hormone in this tick, did not reverse the effects of MK-243 on ovary development. These data suggest that MK-243 may exert its inhibition of egg development more at the level of vitellogenin uptake than vitellogenin synthesis. MK-243 also reversed salivary gland degeneration slightly, probably via its inhibitory effect on 20E-synthesis.

Key Words: Avermectin, Vitellogenesis, 20-Hydroxyecdysone, *Amblyomma hebraeum*, MK-243, ticks

1. Introduction

In ixodid ticks, synthesis of the main yolk protein, vitellogenin (Vg), and its uptake by oocytes, begins within a few days of engorgement on a blood meal. Until recently, little was known about the hormonal regulation of Vg-synthesis in ticks. Current evidence strongly suggests that an ecdysteroid is the vitellogenic hormone in the ixodid ticks, *Ixodes scapularis* [1], *Dermacentor variabilis* [2], and *Amblyomma hebraeum* [3], and the argasid tick, *Ornithodoros moubata* [4]. In *A. hebraeum*, the ecdysteroid, 20-hydroxyecdysone (20E), stimulates Vg-synthesis, but appears insufficient on its own to trigger Vg-uptake (Friesen and Kaufman, submitted manuscript).

Avermectins (AVMs) are a group of broad-spectrum anti-parasitic compounds originally isolated from the bacterium *Streptomyces avermitilis* (reviewed in [5]). Early research suggested that AVMs act as potentiators of γ-aminobutyric acid (GABA) systems in nematodes and arthropods, possibly through an agonistic action on GABA-mediated chloride channels (reviewed in [6]). However, more recent reports indicate that the main mode of action is to modulate glutamate-gated ion channels [7, 8, 9], channels which may also bind GABA [10].

Although treatment of host animals with AVM does not kill or cause detachment of ticks, it does interfere with physiological functions such as molting, feeding to engorgement, and reproduction in *Amblyomma americanum* [11]. AVMs also inhibit oviposition when injected into *A. hebraeum* [12]. The mechanisms behind the latter effects are not known. However, Lunke and Kaufman [13] observed a marked reduction of hemolymph 20E-concentration and inhibition of ovarian development in engorged female *A. hebraeum* following injection of the water-soluble AVM-analogue, MK-243.

The effect of MK-243 might have been due to any combination of the following inhibitions: (1) 20E-synthesis and/or release, (2) Vg-synthesis and/or release into the hemolymph and, (3) uptake of Vg by the ovary. The purpose of this study was to clarify the relative importance of these proposed mechanisms.

2. Materials and Methods

2.1 Ticks

A. hebraeum ticks were maintained in a laboratory colony at 27°C, >95% humidity and in darkness. Ticks were allowed to feed on rabbits, as described by Kaufman and Phillips [14], until they engorged and spontaneously detached.

2.2 *Injections of MK-243 and 20E*

MK-243 was a gift from Merck Sharp and Dohme Research Laboratories. A stock solution of MK-243 (1 mg/ml) in 1.2% saline was stored at -20°C until needed. Just prior to injection, this stock was diluted to 5, 10, and 15 µg/ml in 1.2% NaCl (isosmotic to tick hemolymph). Injected at 1 µl/100 mg body weight (bw), these concentrations of MK-243 corresponded to doses of 50, 100, and 150 ng/g bw. On the day of detachment (day 0), ticks were weighed and MK-243 was injected into the hemocoel through the camerostomal fold (articulation between the scutum and capitulum), using an AGLA micrometer syringe apparatus (Wellcome Reagents Ltd). Ticks were isolated in individual gauze-covered glass vials and stored under colony conditions until 5 or 10 days after injection, at which time hemolymph and tissue samples were collected. Control ticks were injected with 1.2% saline.

To test whether injections of 20E could reverse the effect of MK-243, ticks were treated with 150 ng MK-243/g bw on day 0, and this was followed by 3 bolus injections of 20E (Simes, Milan) on days 1, 3 and 5. Multiple injections were considered necessary because of the rapid rate of catabolism of 20E [15]. 20E stock solutions of (A) 5 mg/ml and (B) 15 mg/ml were prepared in 70% ethanol (EtOH), and diluted to working concentration in 1.2% NaCl immediately prior to injection. The concentration of EtOH in the injected solution was 3%; after injection of 20 µl/g bw, the concentration of EtOH in the body of the tick was estimated to be approximately 0.06%. The resulting doses were 5 µg/g bw and 15 µg/g bw for each bolus injection; these concentrations of 20E are known to stimulate Vg synthesis in partially-fed *A. hebraeum* [3]. Between each injection, ticks were held in the colony incubator. Ticks were dissected on day 10 post-engorgement, and samples of ovary (and hemolymph where possible) were collected.

2.3 Collection of hemolymph and ovary samples

On the day of dissection (day 5 or day 10 post-injection), ticks were secured ventrally to a petri dish with cyanoacrylate glue and refrigerated for 15 min. Cooling ticks prior to hemolymph collection inhibits gut contraction, thus reducing the chance of breaking the gut and contaminating the hemolymph [16]. Incisions (1-2 mm long) were made in the integument with a microscalpel. The exuding hemolymph was collected with volumetric glass capillary tubes and diluted 1:4 in phosphate-buffered saline (PBS; 35 mM NaH_2PO_4, 60 mM Na_2HPO_4, 150 mM NaCl, pH 7.0). Any sample contaminated with gut contents was discarded. Hemolymph samples were stored at -70°C until further analyzed.

Following hemolymph collection, ticks were flooded with a modified Hank's balanced saline (200 mM NaCl, 8.9 mM D-glucose, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.44 mM KH_2PO_4, 0.35 mM Na_2HPO_4, 27 μM phenol red, pH 7.2), and the dorsal cuticle removed. Ovaries were dissected out, and length of the long axis of the eight apparently largest ovoid oocytes was measured using an ocular micrometer fitted to a compound microscope. The mean value for the eight oocytes was recorded for each tick. Ovaries were then gently blotted, weighed, and homogenized in 100 μl PBS per 30 mg ovary. Ovary homogenates were stored at -70°C until further analyzed.

2.4 ELISA for Vg and Vt

Hemolymph Vg and ovary vitellin (Vt) were quantified using an indirect competitive ELISA as described by Friesen and Kaufman [3]. Partially purified Vt from day 10 ovaries was used as the standard for determining the concentration of unknown samples. Briefly, wells of a 96-well microtitre plate were coated with1 μg partially-purified Vt, and a mixture of anti-Vg antibodies, plus either known concentrations of Vt or unknown samples, added to each well. After incubation, the amount of antibody binding to the plate-bound Vt was quantified using an alkaline phosphatase (AP)-linked goat anti-rabbit secondary antibody (BioRad) and an AP colour substrate kit (p-Nitropheynlphosphate and diethanolamine kit; BioRad). The colour reaction was quantified by measuring the absorbance of each well at 405 nm using a microtitre plate

reader (Bio-Tek). The sensitivity of this ELISA to *A. hebraeum* Vg is approximately 5 ng of Vt-equivalents

2.5 Assay for salivary gland degeneration

As 20E triggers salivary gland degeneration [16], and because MK-243 reduces hemolymph ecdysteroid concentration [13], we also measured salivary gland function in this study using the technique of Harris and Kaufman [17]. Briefly, paired salivary glands were excised from each tick 5 or 10 days post-engorgement, and the main ducts ligated using very fine strands of surgical silk thread (Dermalon®; Davis and Geck). The glands were gently blotted, weighed to the nearest 10 µg and incubated in medium TC 199 (Gibco) containing 10 µM dopamine (Sigma) for 10 min, blotted again, and weighed. As dopamine stimulates salivary fluid secretion [17], the net weight increase is a direct measure of fluid secretory competence; loss of fluid secretory competence compared to controls is thus a quantitative measure of salivary gland degeneration. The wet, silk thread weighed less than the sensitivity of the balance (10 µg).

2.6 Photography

Whole ticks, ovaries, and salivary glands were photographed using a Nikon DXM1200 digital camera attached to a dissecting microscope. Digital images were re-touched for publication using Adobe Photoshop 4.0 software.

2.7 Statistical analysis

Results are reported as mean ± SEM (n). Statistical analysis was done using Statview 4.02. Differences among treatments were analyzed using a one-way analysis of variance (ANOVA). Statistical significance is indicated as follow: (*) $0.01<P<0.05$; (**) $0.001<P<0.01$; (***) $P<0.001$.

3. Results

3.1 Effects of MK-243 on engorged ticks

Except for a single tick at 50 ng MK-243/g bw, the doses of MK-243 used in this study did not kill engorged ticks by day 5 (Table 1). By day 10 post-engorgement, mortality increased only slightly with dose of MK-243, peaking at 11% at the highest dose (150 ng MK-243/g bw). However, ticks treated with MK-243 appeared bloated, had splayed legs, did not move, and displayed much shallower dorsal ridges than normal healthy ticks (Fig. 1), suggesting that the major dorso-ventral muscles and leg muscles were paralyzed.

Ovary weight in control ticks rose 4.7-fold between days 5 and 10 (Fig. 2A, control). In contrast, ovary weights of ticks treated with 150 ng MK-243/g bw were significantly smaller, being only 32% of control on day 5 and 19% of control On day 10 (Fig. 2A). On day 5, mean oocyte length of ticks treated with 150 ng MK-243/g bw was 60% of the control value (Fig. 2B); likewise, on day 10, oocyte length of ticks treated with 150 ng MK-243/g bw was 51% of the control value.

Total Vt content of the ovary in day 10 ticks treated with 150 ng MK-243/g bw was reduced by 91% compared with saline injected control ticks (Fig. 2C, bars). Vt as % ovary weight dropped 60% at 100 ng MK-243/g bw, with no further decline at 150 ng MK-243/g bw (Fig. 2C, open circles). Hemolymph Vg concentration on days 5 and 10 was not significantly inhibited by MK-243 (150 ng/g bw) (Fig. 2D). For both days, however, the variability was high.

Ovaries of day 5 ticks treated with MK-243 showed numerous regions where oocytes were in the previtellogenic growth phase, but in general the oocytes were smaller than those of controls, with few having begun Vg-uptake (Fig. 3A and 3B). By day 10, MK-243 treated ovaries contained some clusters of oocytes at advanced stages of Vg-uptake (Fig. 3D), whereas Vg-uptake in day 10 controls occurred in oocytes along the entire length of the ovary (Fig. 3C).

3.2 Effects of 20E on MK-243 treated ticks

Because MK-243 inhibits 20E hemolymph titers [13], and because 20E is probably the vitellogenic hormone in *A. hebraeum* [3], we tested whether injections of 20E could reverse the inhibitory effects of MK-243 on the reproductive system. In general, 20E (5 or 15 µg/g bw) did not reverse the inhibitory effect of MK-243 (150 ng/g bw) on ovary weight (Fig. 4A), mean oocyte length (Fig. 4B), or ovary Vt-content (Fig. 4C). Note, however, that the mortality of ticks injected with 20E was substantially higher than the mortality of ticks treated with MK-243 alone, reaching 40% at a dose of 15 µg 20E/g bw (Table 2).

3.3 Effects of MK-243 on salivary gland weight and salivary fluid secretory competence

There were no significant differences in salivary gland weight of ticks treated with MK-243 compared with control ticks on day 5 post-engorgement (Fig. 5A). However, doses of 100 and 150 ng MK-243/g bw increased salivary gland weight significantly by day 10 (Fig. 5A). Because the salivary glands degenerate significantly over the first 4 days of engorgement [17], salivary fluid secretory competence was generally low in both day 5 and 10 control ticks (Fig. 5B). Doses of 50 ng MK-243/g bw and 150 ng MK-243/g bw, caused day 5 salivary glands to take up significantly more fluid than control ticks; this trend was not seen on day 10, however (Fig. 5B).

Salivary glands of MK-243 treated ticks generally appeared more robust than those of control ticks. Ten days after engorgement, salivary glands of controls were extremely fragile, and had a wispy appearance under the dissecting microscope compared to MK-243 treated tick salivary glands (Fig. 6).

4. Discussion

This study indicates that MK-243 inhibits egg development primarily at the level of Vg-uptake by the oocyte. First, even though MK-243 reduces hemolymph ecdysteroid titer by approximately 90% [13], multiple injections of 20E did not reverse the action of MK-243 (Fig. 4). Second, MK-243 did not significantly reduce hemolymph Vg-concentration (Fig. 2D). Finally, ovary weight (Fig. 2A) and vitellin content of the ovary (Fig. 2C) were the most affected by MK-243. However, it is not yet possible to entirely exclude an effect of MK-243 on Vg-synthesis. For example, if synthesis and uptake of

Vg were inhibited to a similar degree, this would result in little or no change in Vg-concentration in the hemolymph, as was observed here (Fig.2D). On the other hand, treatment with 20E should then have resulted in an increase in Vg-concentration of the hemolymph, but this was not the case (results not shown). This matter might be resolved by measuring the effect of MK-243, with and without 20E, on radiolabelled amino acid accumulation into Vg.

The effects of MK-243 on the ovary were much more noticeable on day 10 than day 5. The bulk of ovary growth is due to Vg-uptake, which occurs between days 4 and 16 post-engorgement [3]. By day 5, most of oocyte growth is due to the previtellogenic phase of development [18, 19]. This may explain why MK-243 showed less effect by day 5 than day 10 (Fig. 2B). Similar results were observed in mosquitoes, where oocyte growth due to Vg-uptake was inhibited after ivermectin treatment [20].

Oocyte length was not an accurate measure for testing the effect of MK-243 in *A. hebraeum*. MK-243 reduced the Vt content of ovaries by up to 81% (Fig. 2C), whereas oocyte length was reduced by only 40% (Fig. 2B). It is clear from Fig. 3 that many fewer oocytes accumulated Vg in MK-243 treated ticks compared to control ticks. But at least a few clusters of oocytes accumulated Vg even at the highest dose of MK-243. The fact that our index of oocyte size was based on measuring the eight apparently largest oocytes accounts for why this index was less sensitive. It is interesting that those oocytes which did accumulate Vg seemed to be clustered, rather than randomly distributed (Fig. 3D). The reason for this is unknown, but might be that these oocytes were at a more advanced stage of development at the time of treatment, or that some autosynthesis of Vg by the

ovary occurred, as previously suggested for the ixodid tick, *Rhipecephalus sanguineus* [21] or that, for some other reason, they escaped the effects of MK-243.

However, Because MK-243 inhibits 20E-synthesis [13], we hypothesized that it might also inhibit salivary gland degeneration. MK-243 did cause a small increase in fluid secretory competence, at least on day 5 (Fig. 5B), although secretory competence did not approach the values expected for salivary glands from partially-fed *A. hebraeum*, 5-10 days post-removal (about 3 mg/gland /10 min [12, 22]). This is probably because although MK-243 reduces hemolymph ecdysteroid concentration by approximately 90% [13], the residual concentration (about 50 ng/ml) would still be above the threshold for some degree of salivary gland degeneration (30 ng/ml; [23]).

In summary, this study indicates that MK-243 acts primarily by inhibiting Vg-uptake by the oocytes. The mechanism of this inhibition remains to be determined.

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Fig. 1. Appearance of engorged female *A. hebraeum* 10 days after injection of (A) 1.2% NaCl or (B) 150 ng MK-243/g bw. The cuticular ridges (white arrowheads) are caused by the contraction of the dorso-ventral muscles. Note that the ridges are much shallower in the MK-243 treated tick and that the legs are splayed (indices of paralysis) compared to the control.

Fig. 2. Effect MK-243 on ovary development in *A. hebraeum*, 5 days (light grey bars) or 10 days (dark grey bars) post-engorgement. MK-243 was injected on the day of engorgement (day 0). (A) Ovary weight as % engorged body weight as measured on day 0; (B) mean length of 8 of the largest oocytes; (C) total Vt-content of ovary; (D) hemolymph Vg-concentration (reported as 'Vt-equivalents', see Materials and Methods). For all panels, data are reported as mean ± SEM (n). Statistical significance is indicated as follows: (*) $0.01<P<0.05$; (**) $0.001<P<0.01$; (***) $P<0.001$.

Fig. 3. Effect of MK-243 on appearance of the ovary. Ovaries are from (A) day 5 post-engorgement saline injected control, (B) day 5, 100 ngMK-243/g bw, (C) day 10, saline injected control (D) day 10, 100 ng MK-243/g bw. Some of the regions containing vitellogenic oocytes are indicated by white brackets. Asterisks (*) indicate a few regions where oocytes are toward the end of the great cytoplasmic growth phase, but that have not yet accumulated much Vg (see Discussion).

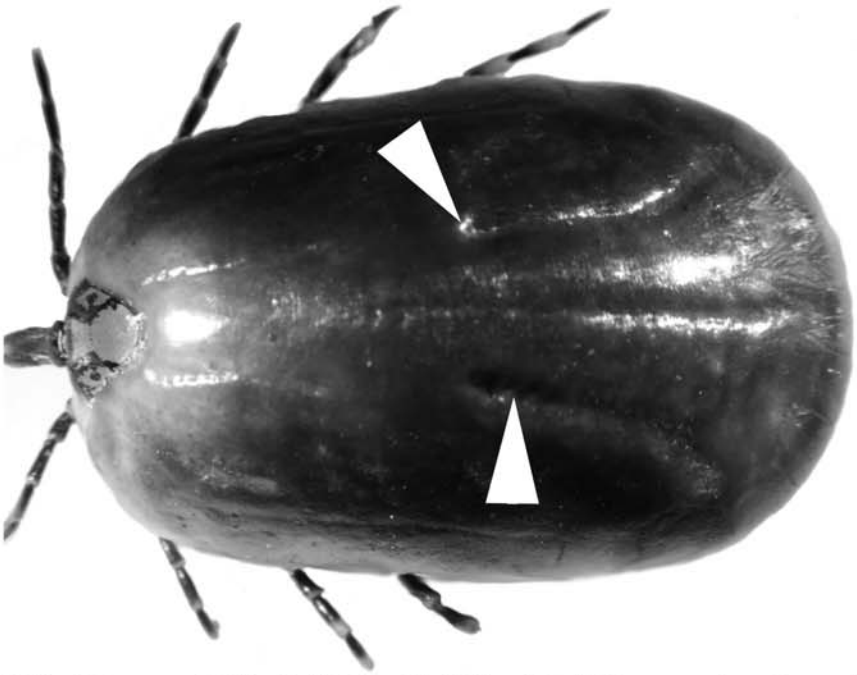
Fig. 4. Inability of 20E to reverse the effects of MK-243 in engorged *A. hebraeum*. MK-243 (150 ng/g bw) or saline control was injected on day 0. Bolus injections of 20E were

given on days 1, 3, and 5 post-engorgement, and ovaries collected on day 10. (Control): ticks that received no injection; (EtOH): ticks that received EtOH (final concentration 0.06%) in 1.2% NaCl on days 1, 3, and 5; (MK-243): ticks that received 150 ng MK-243/g bw followed by ethanol/saline injections (days 1, 3, and 5). (A) Ovary weight as % bw; (B) mean length of 8 of the longest oocytes; (C) total Vt-content of the ovary. Data are reported as mean ± SEM (n). Significant differences are indicated as in the legend to Fig. 2.

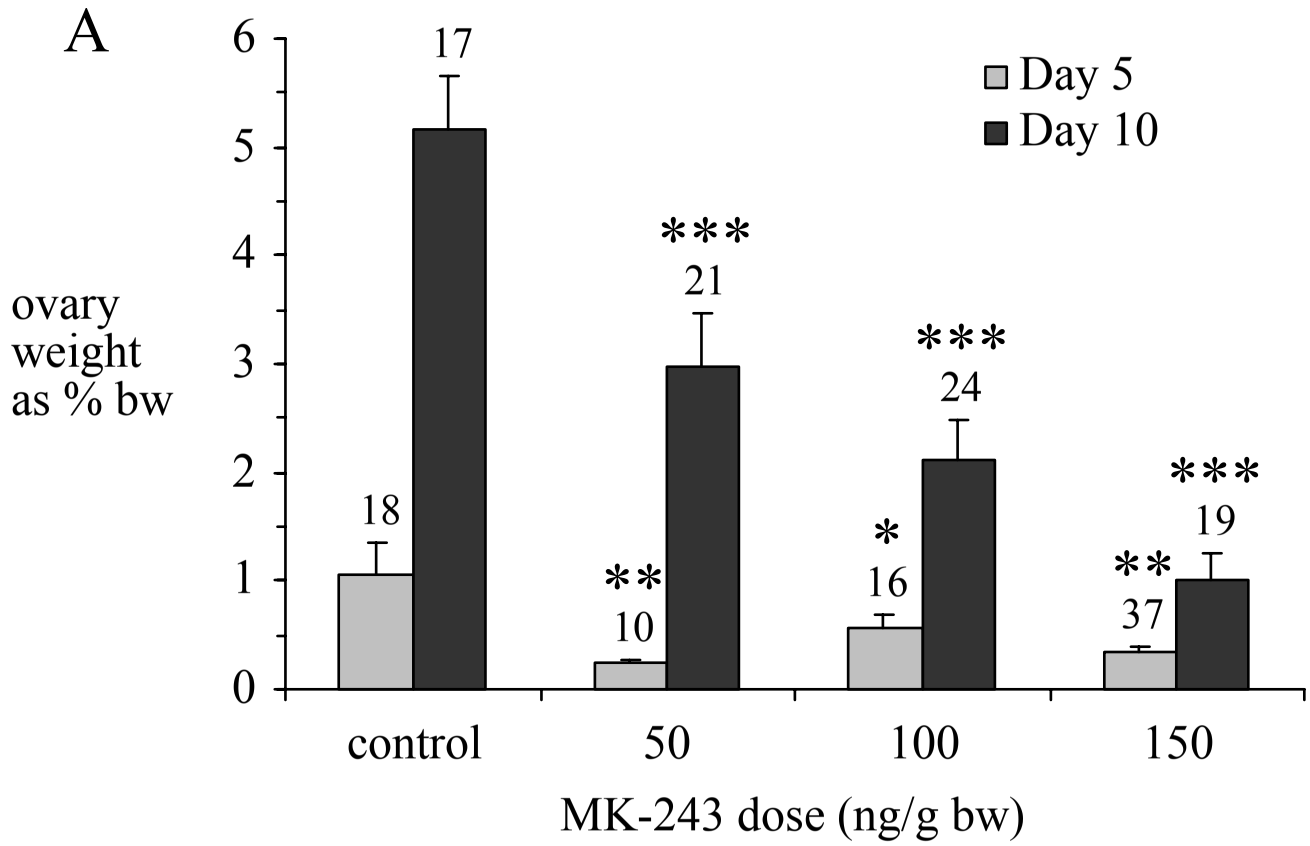
Fig. 5. Effect of MK-243 on salivary gland (SG) weight (A) and fluid secretory competence (B) 5 and 10 days post-engorgement. Ticks received a single injection of 1.2% NaCl (control) or MK-243 (50, 100, or 150 ng/g bw) on the day of engorgement. Data are reported as mean ± SEM (n). Significant differences are indicated as in the legend to Fig. 2.

Fig. 6. Effect of saline injection (A), or 150 ng MK-243g bw (B), on appearance of salivary glands of *A. hebraeum* 10 days post-engorgement. Note the wispy appearance of the control salivary gland versus the more robust appearance of the MK-243 treated gland.

B) Day 10 control



B) Day 10 MK-243 (150 ng/g bw)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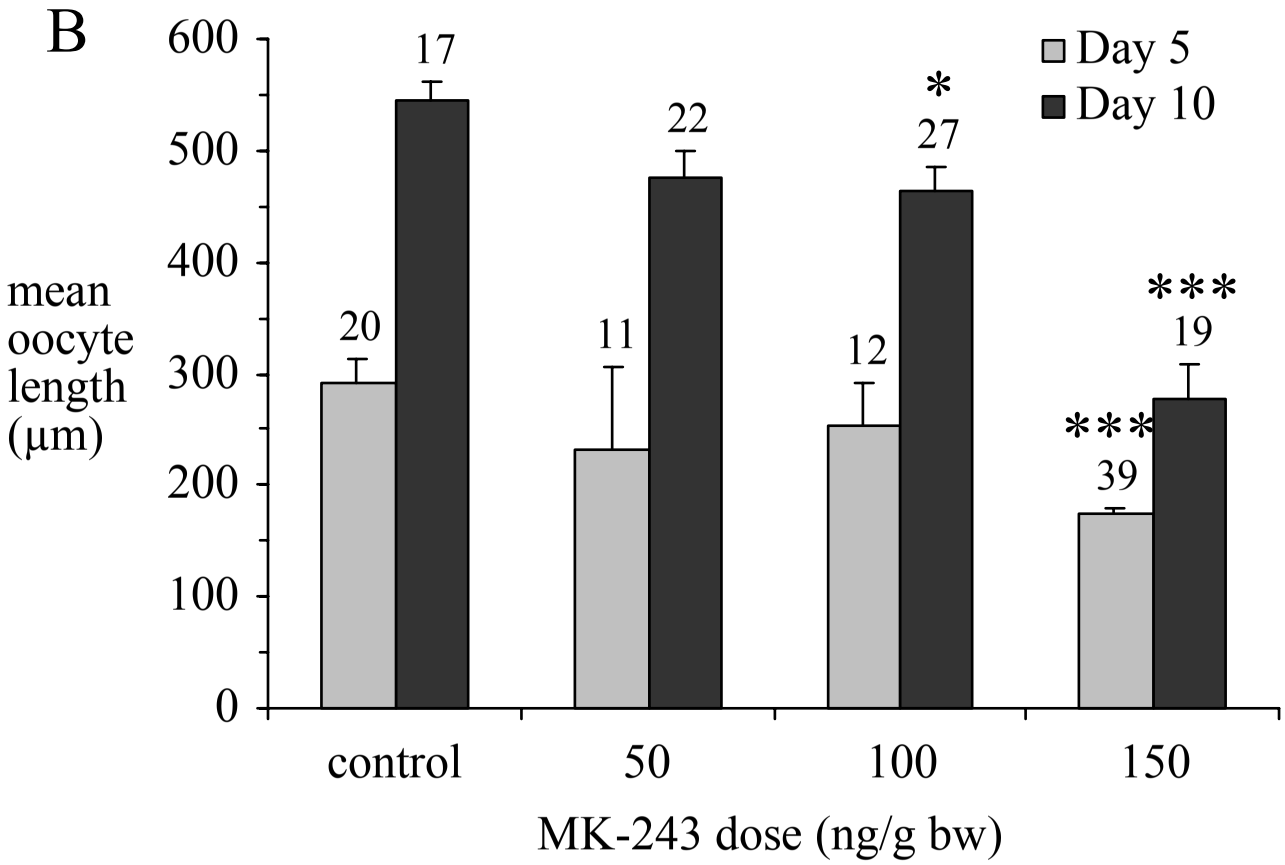
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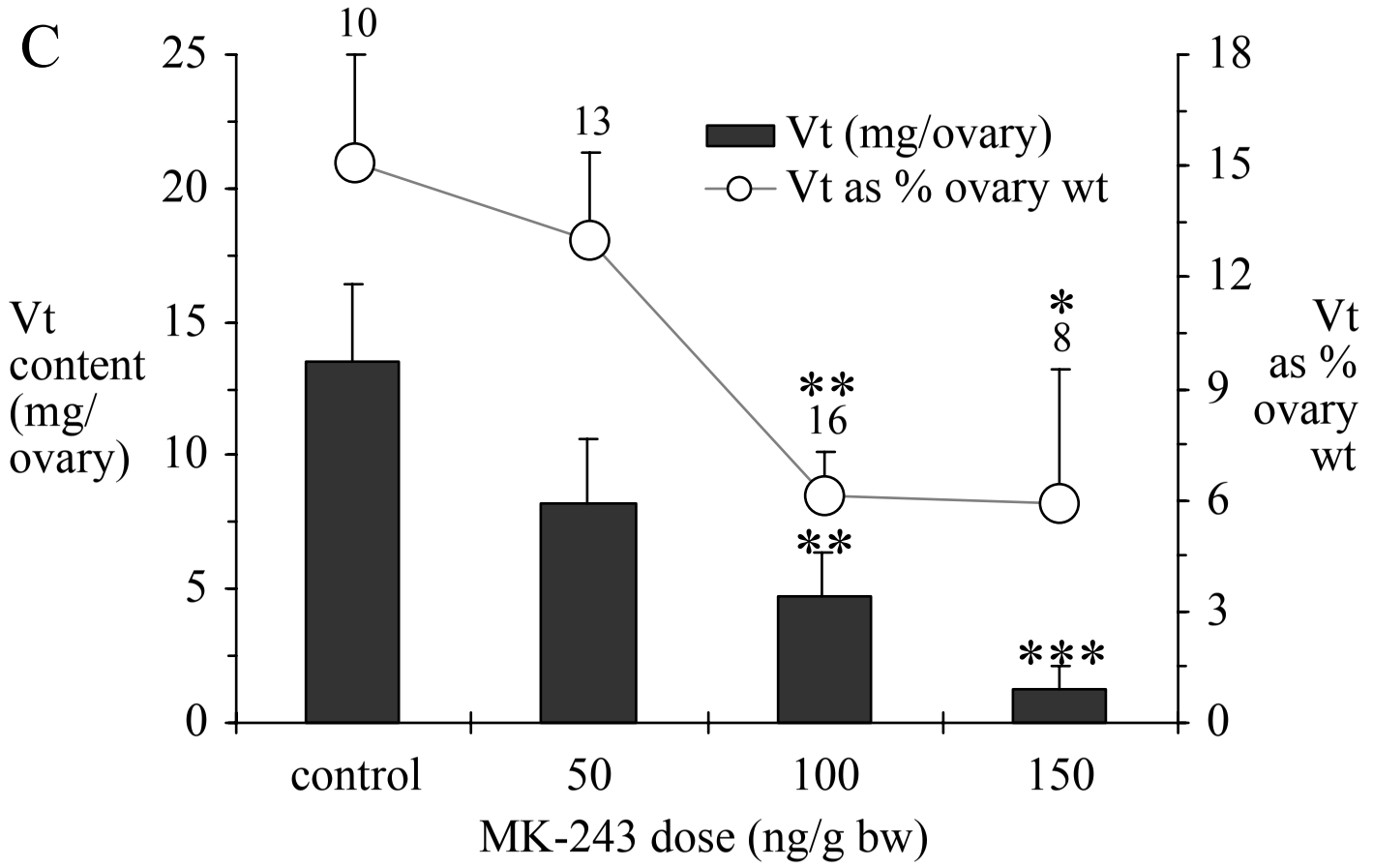
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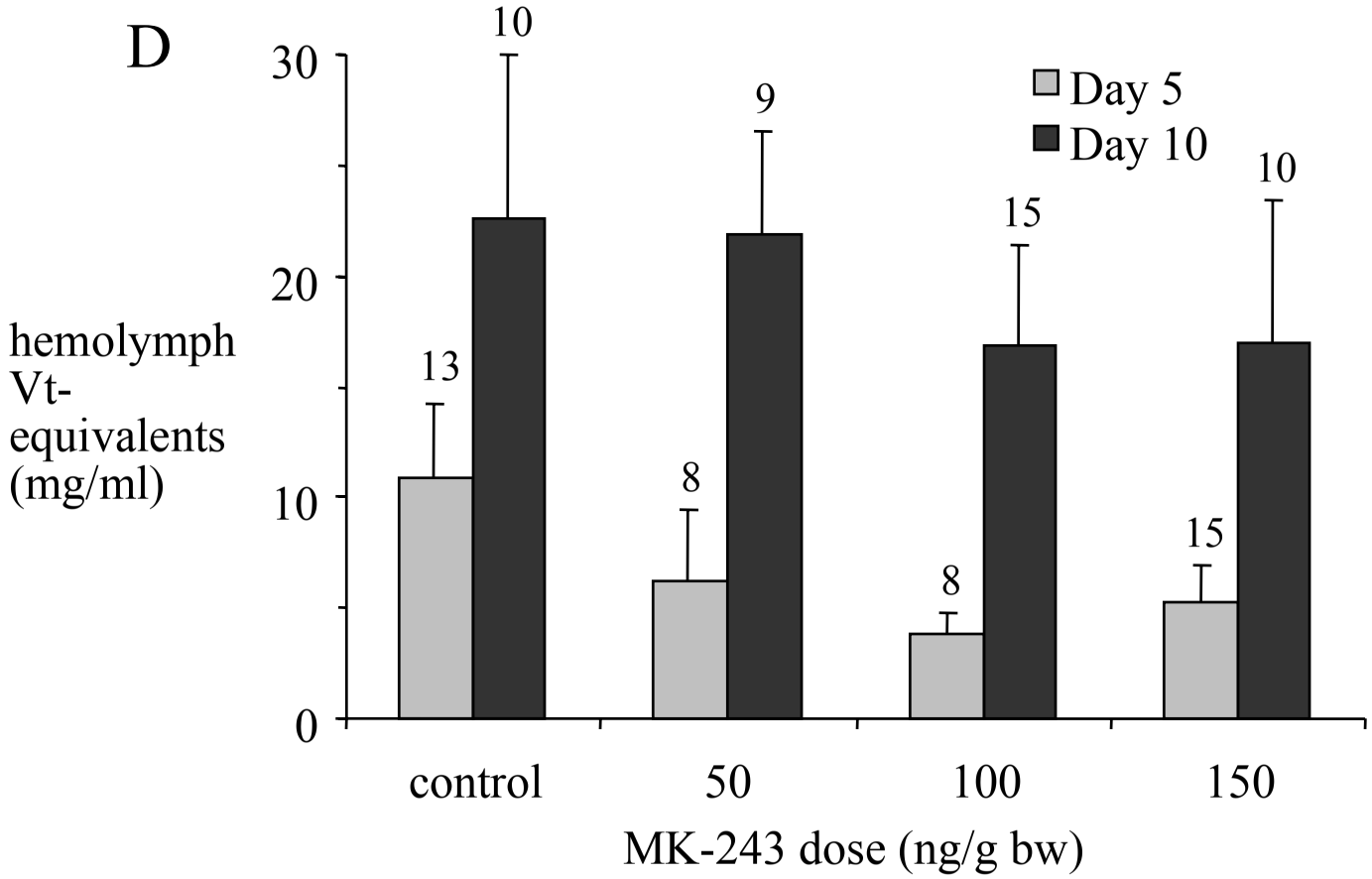
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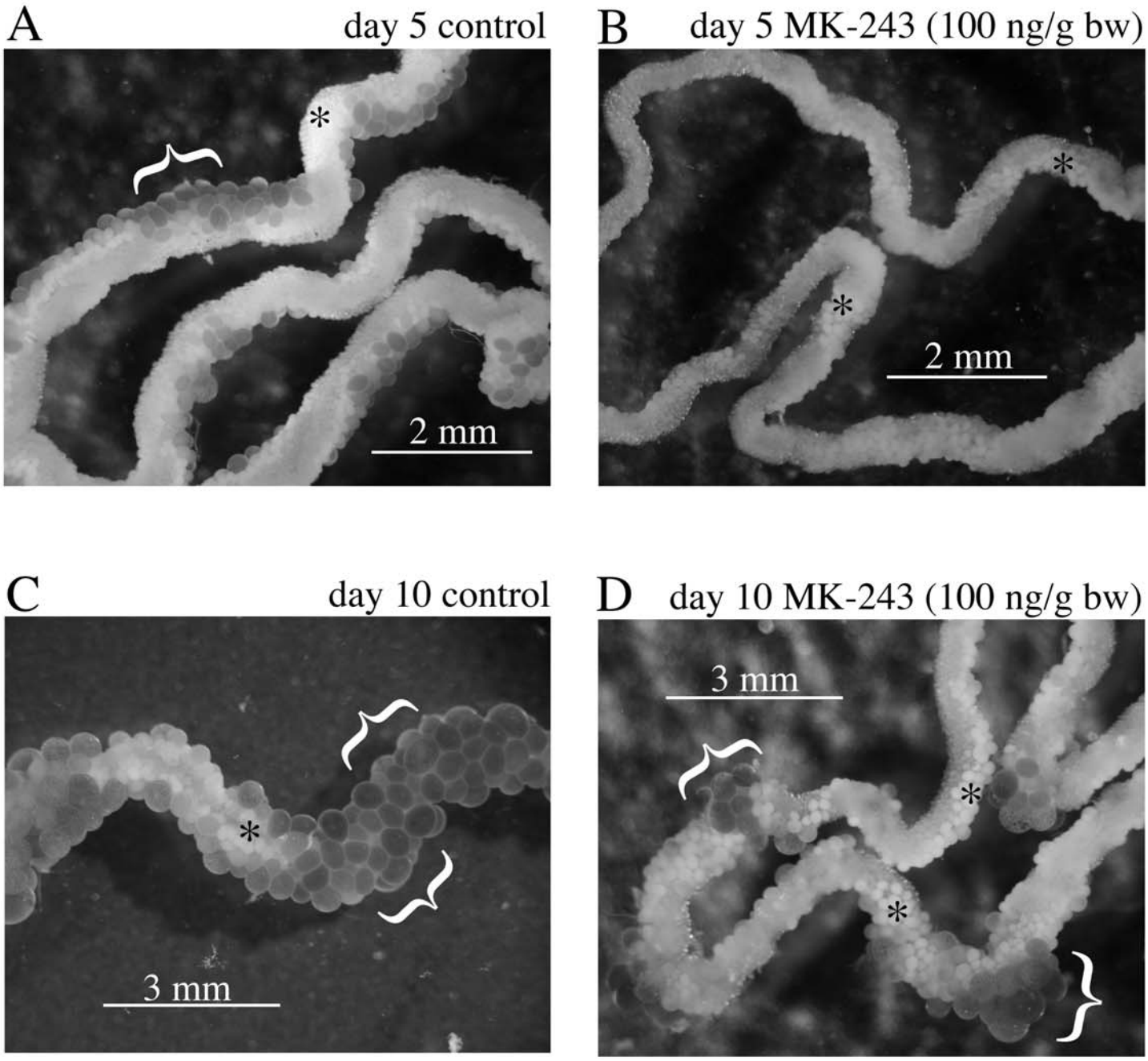
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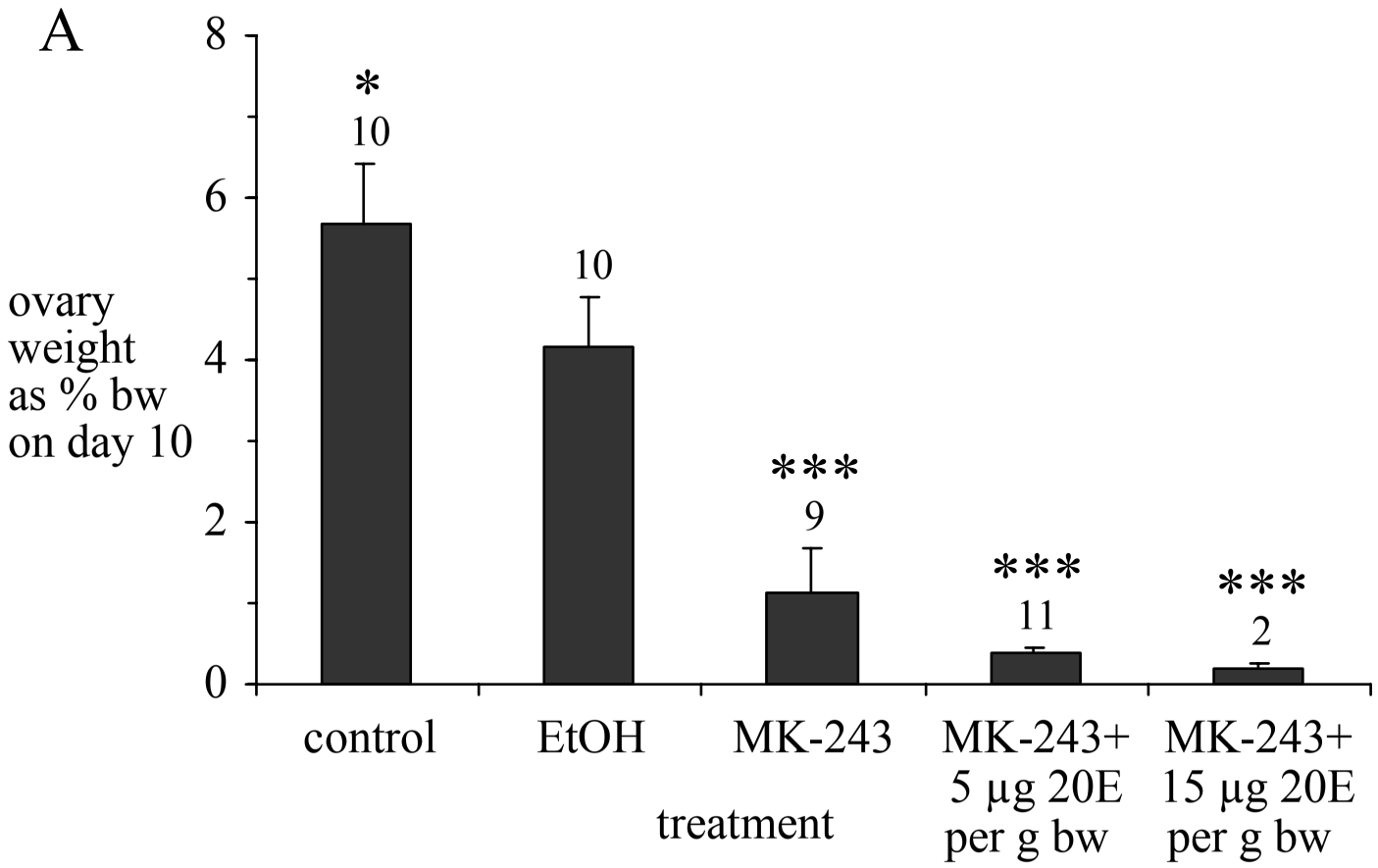
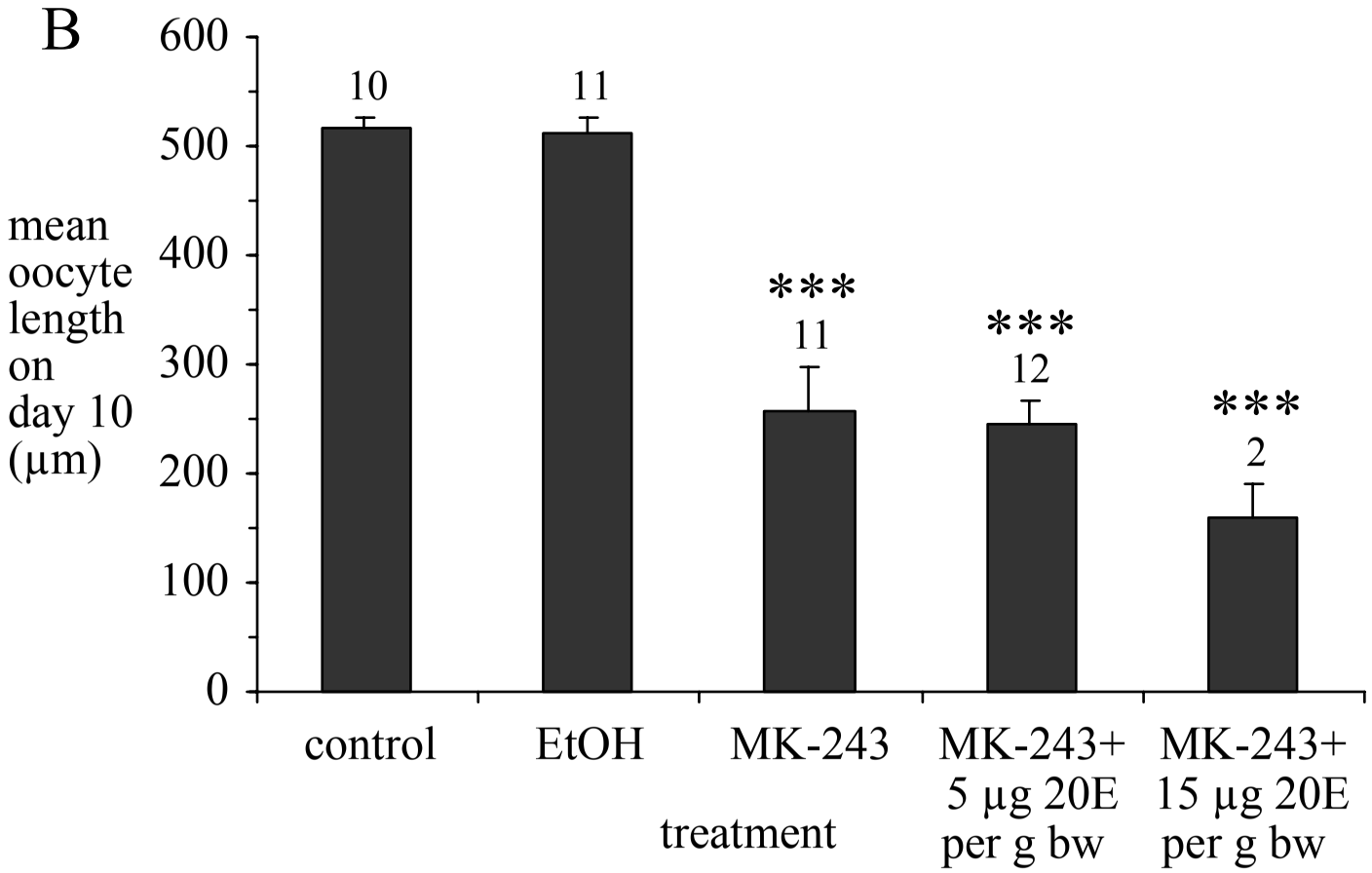
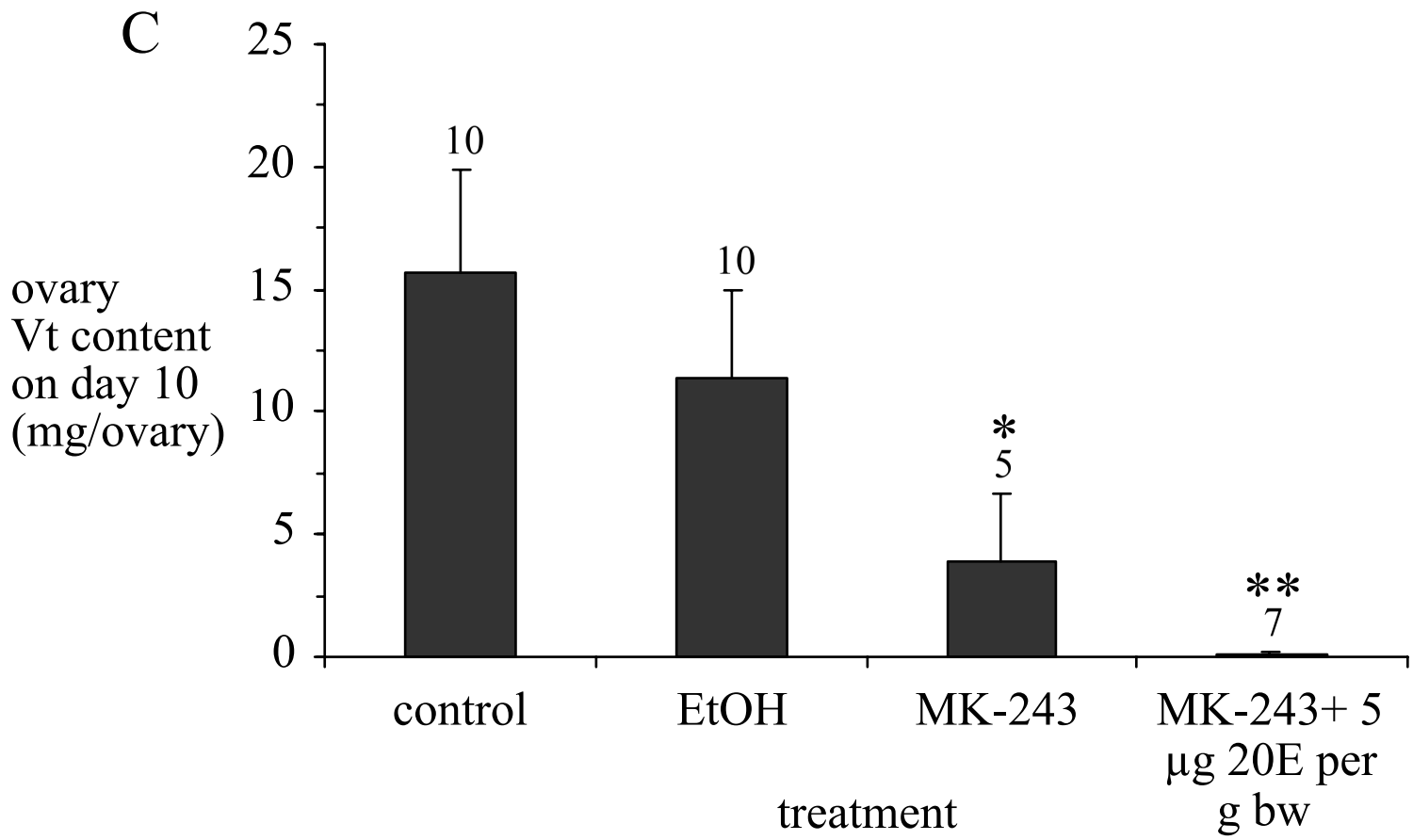
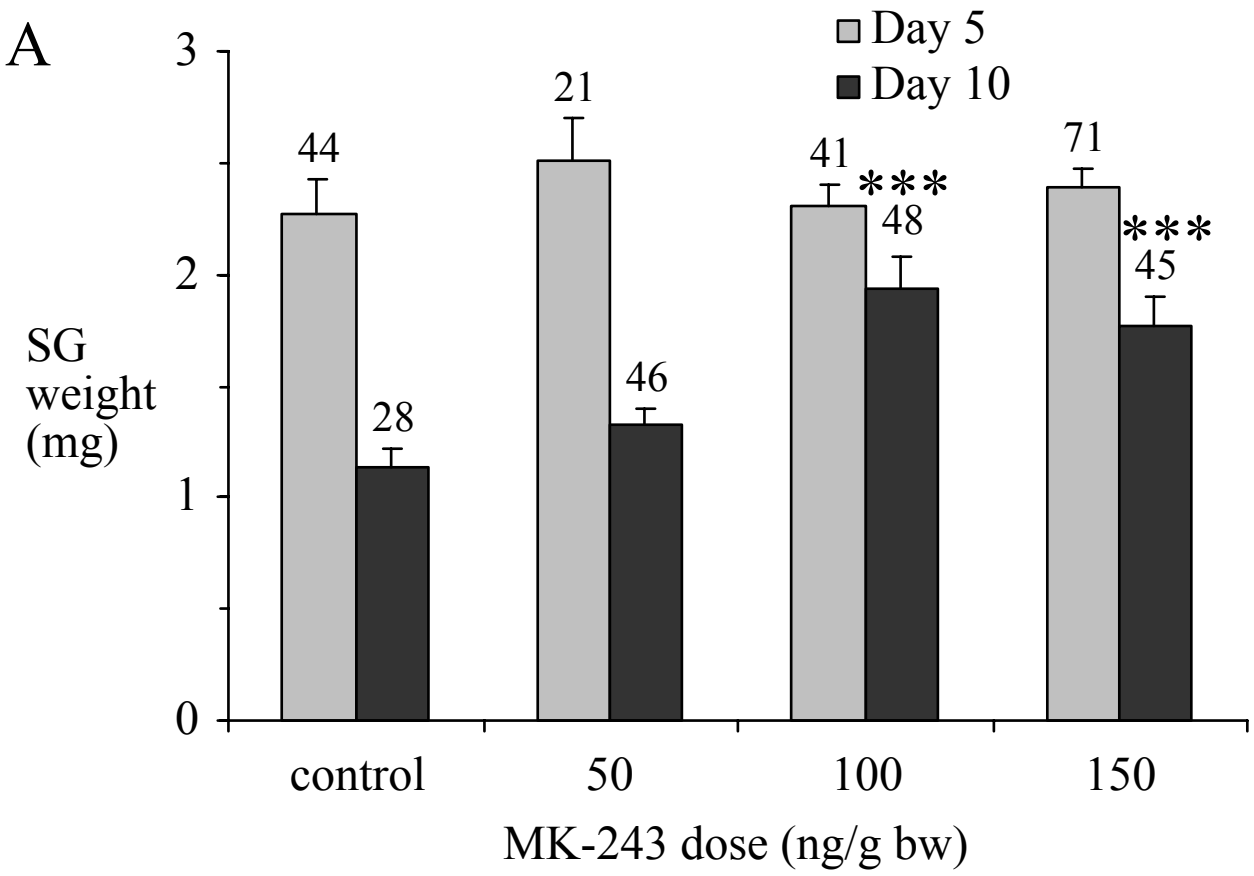
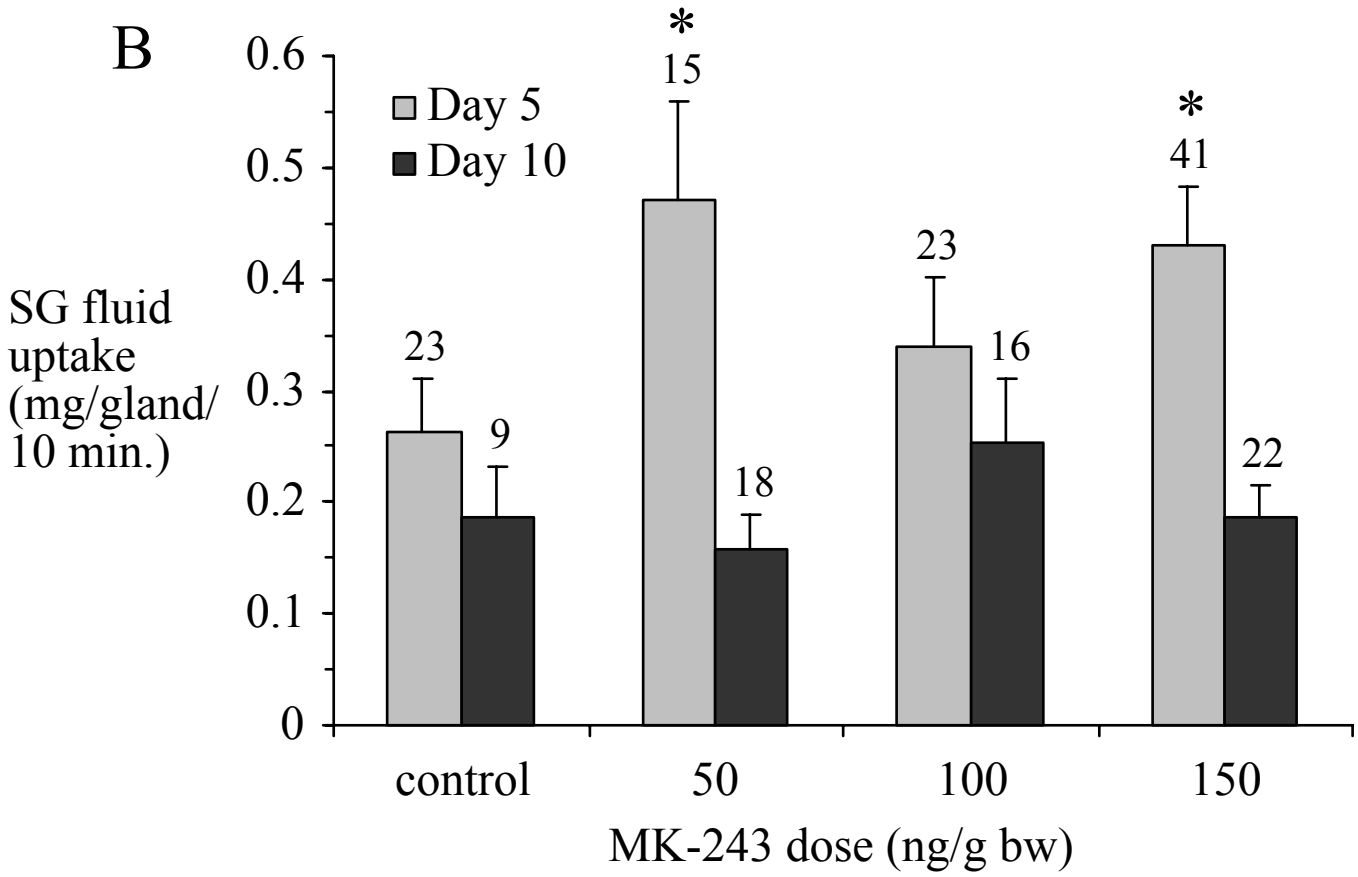
2 mm
B
day 5 MK-243 (100 ng/g bw)
2 mm
C
day 10 control
3 mm
D
day 10 MK-243 (100 ng/g bw)
3 mm


A
8
6
4
2
0
ovary
weight
as % bw
on day 10
*
10
10
***
9
***
11
***
2
control
EtOH
MK-243
MK-243+
5 µg 20E
per g bw
MK-243+
15 µg 20E
per g bw
treatment

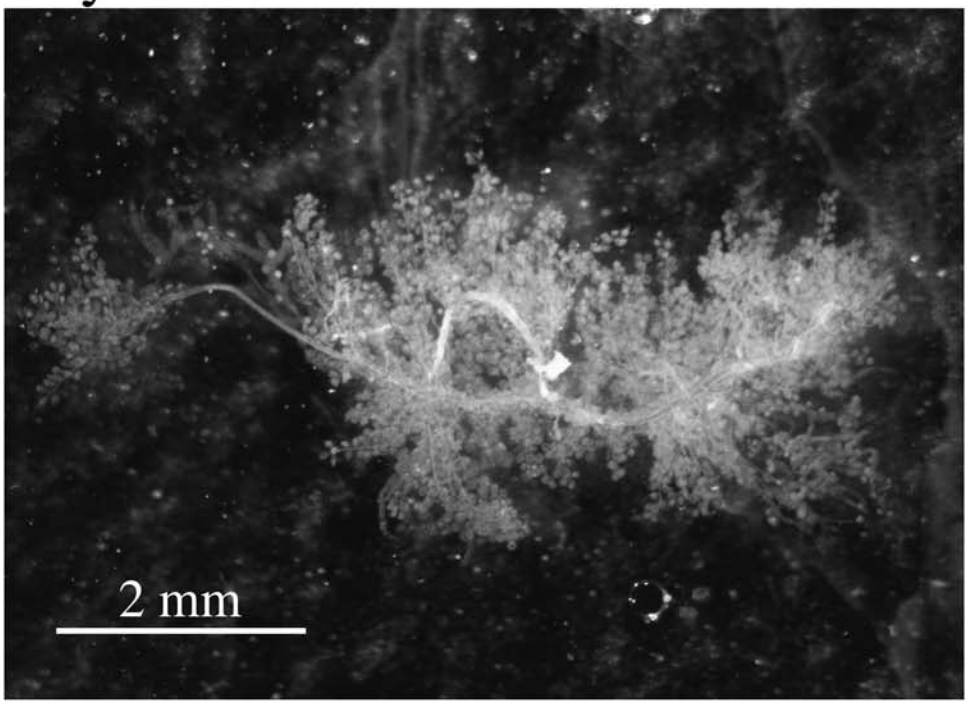

B
600
500
400
300
200
100
0
mean oocyte length on day 10 (µm)
10
11
***
11
***
12
***
2
control
EtOH
MK-243
MK-243+ 5 µg 20E per g bw
MK-243+ 15 µg 20E per g bw
treatment


C
25
20
15
10
5
0
ovary
Vt content
on day 10
(mg/ovary)
10
10
*
5
**
7
control
EtOH
MK-243
MK-243+ 5
µg 20E per
g bw
treatment


A
Day 5
Day 10
SG weight (mg)
3
2
1
0
44
28
21
46
41
48
***
71
45
***
control
50
100
150
MK-243 dose (ng/g bw)


B
SG fluid uptake (mg/gland/ 10 min.)
0.6
0.5
0.4
0.3
0.2
0.1
0
Day 5
Day 10
23
9
*
15
18
23
16
*
41
22
control
50
100
150
MK-243 dose (ng/g bw)

Day 10 control



Day 10 MK-243 (150 ng/g bw)

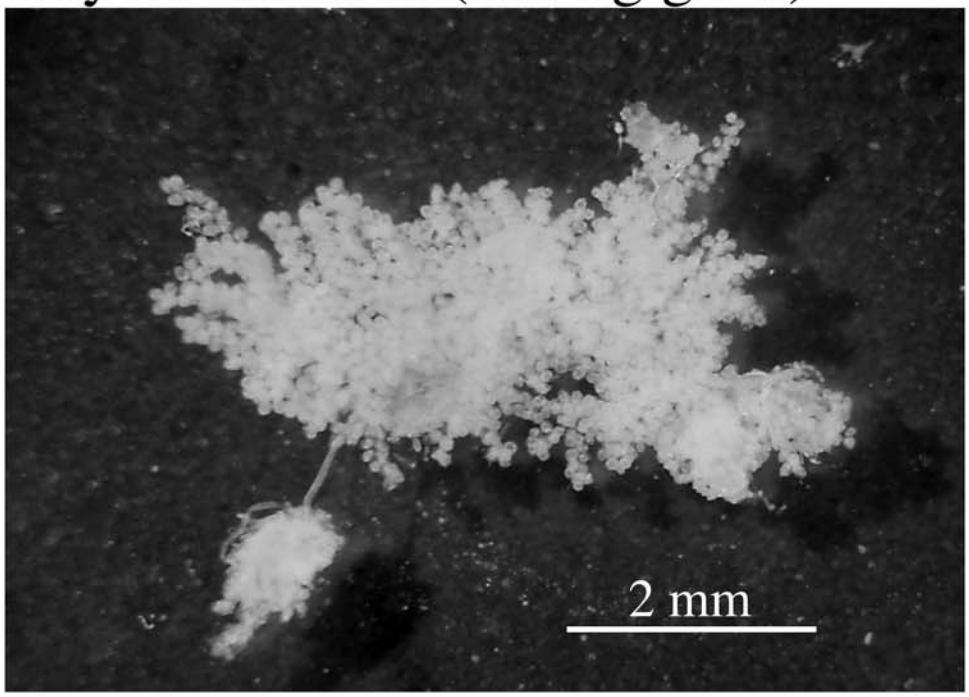


Table 1: Mortality of MK-243 treated engorged female *A. hebraeum* on day 5 or 10 post-engorgement.

	[MK-243] ng/g bw							
	0		50		100		150	
	% mortality	n	% mortality	n	% mortality	n	% mortality	n
Day 5	**0**	36	**7.1**	14	**0**	34	**0**	71
Day 10	**3.7**	27	**6.4**	47	**7.9**	38	**10.9**	55

Table 2: Mortality of MK-243/20E treated engorged female *A. hebraeum* on day 10 post-engorgement.

Treatment									
untreated		EtOH		MK-243 (150 ng/g bw)		MK-243 + 20E (5 µg/g bw)		MK-243 + 20E (15 µg/g bw)	
% mortality	n	% mortality	n	% mortality	n	% mortality	n	% mortality	n
0	16	**0**	21	**0**	20	**14.8**	27	**40**	10