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Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the *Defensin* gene

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***Anopheles gambiae*, the major vector of human malaria parasite, is an important insect model to study vector–parasite interactions. Here, we developed a simple *in vivo* double-stranded RNA (dsRNA) knockout approach to determine the function of the mosquito antimicrobial peptide gene *Defensin*. We injected dsRNA into adults and observed efficient and reproducible silencing of *Defensin*. Analysis of the knockdown phenotype revealed that this peptide is required for the mosquito antimicrobial defense against Gram-positive bacteria. In contrast, in mosquitoes infected by *Plasmodium berghei*, no loss of mosquito viability and no significant effect on the development and morphology of the parasite midgut stages were observed in the absence of Defensin. We conclude that this peptide is not a major antiparasitic factor in *A. gambiae in vivo*. Our results open new perspectives for the study of mosquito gene function *in vivo* and provide a basis for genome-scale systematic functional screens by targeted gene silencing.**

INTRODUCTION

Anopheles gambiae is the most important vector for *Plasmodium falciparum* malaria in sub-tropical Africa and thus a critical link in the transmission cycle of one of the most serious infectious diseases of humanity (Greenwood and Mutabingwa, 2002). In recent years, this mosquito has been studied intensively by the methods of molecular and cell biology, with a special emphasis on innate immune mechanisms possibly implicated in limiting the load of parasite transmission. Such studies promise to advance rapidly when the completion of the *A. gambiae* genome sequencing reveals the universe of genes upon which mosquito immunity resides (Hoffman *et al.*, 2002). Despite the existence of a good microsatellite-based genetic map (Zheng *et al.*, 1996) and robust techniques for germ-line transgenesis (Catteruccia *et al.*, 2000; Grossman *et al.*, 2001), inherent limitations of mosquito stock maintenance hinder the traditional methods for gene function analysis, such as large mutagenesis screens, fine-scale gene mapping and transgenic analysis. The double-stranded RNA (dsRNA) interference is potentially adaptable to systematic reverse genetic screens (Gonczy *et al.*, 2000), and we have shown recently that it can be used to assess gene function in cultured mosquito cells (Levashina *et al.*, 2001). Here, we demonstrate that dsRNA can also be used to disrupt essential gene function in the whole mosquito.

When dsRNA is taken up by cells, it is cleaved into small interfering fragments that can trigger specific degradation of the endogenous target mRNA (Zamore *et al.*, 2000). *Caenorhabditis elegans* is the only multicellular organism where the direct injection of dsRNA in the animal has been demonstrated to result in gene silencing throughout development (Fire *et al.*, 1998). In *Drosophila*, dsRNA injection in embryos is widely used for assessing gene function in early development, but in adult flies RNA interference is routinely mediated by the expression of hairpin dsRNA in transgenic strains (St Johnston, 2002), a technique that would be very difficult to apply systematically in mosquitoes. Instead, we have opted for a rapid and direct approach, intrathoracic injection of dsRNA in adult *A. gambiae*, to bring about efficient and reproducible silencing of the gene encoding the antimicrobial peptide, Defensin, which is encoded by a single gene in the *A. gambiae* genome.

So far, three antimicrobial peptides have been characterized in *A. gambiae*, Defensin, Cecropin and Gambicin, which are produced by the fat body and hemocytes and secreted into hemolymph upon immune challenge (Richman *et al.*, 1996; Vizioli *et al.*, 2000, 2001a). These polypeptides exhibit bactericidal and/or fungicidal activities *in vitro* and are thought to constitute the first line of defense against microbial infections

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(reviewed in Dimopoulos *et al.*, 2001; Hoffmann and Reichhart, 2002). Related peptide families exist in vertebrates and are mostly expressed by epithelia and leukocytes, acting locally to limit bacterial infection (reviewed in Lehrer and Ganz, 2002). A knockout mouse lacking the Defensin-like peptide Cathelicidin has been shown recently to be susceptible to necrotic skin infections caused by Gram-positive Group A streptococci (Nizet *et al.*, 2001). In *Drosophila*, the inactivation of two major regulatory signalling pathways, *Toll* and *Imd*, has been used to turn off large groups of immune genes, including the antimicrobial peptide genes, and to associate them collectively with *in vivo* antifungal and antibacterial functions, respectively (Lemaitre *et al.*, 1996). However, no loss-of-function mutants for individual antimicrobial peptide genes have been reported as yet.

The expression of *Defensin* is predominantly induced in the mosquito fat body shortly after bacterial challenge. It is also induced locally in the midgut and salivary gland epithelia upon invasion by malaria parasites, suggesting that Defensin may have a broad role in the defense against both microbes and parasites (Richman *et al.*, 1996, 1997). This presumption was supported by *in vitro* tests of antiparasitic activity and by injection studies in *Aedes* mosquitoes infected by avian malaria (Shahabuddin *et al.*, 1998). However, rigorous conclusions about Defensin function *in vivo* require analysis by a loss-of-function approach in the intact mosquito.

RESULTS AND DISCUSSION

To knock down the *Defensin* gene expression, we injected 1- to 2-day-old females with dsRNA corresponding to the genes for either Defensin (*dsDEF*) or green fluorescent protein (*dsGFP*) as a control and allowed the mosquitoes to recover for 4 days. The dsRNA-treated mosquitoes were then challenged with *Escherichia coli*, and the presence/absence of the *Defensin* transcripts was monitored from day 1 to 8 by RNA blotting and RT–PCR (Figure 1). In six independent experiments, the injection of either Gram-negative *E. coli* or Gram-positive *Staphylococcus aureus* induced the expression of *Defensin* mRNA in non-treated control mosquitoes (Figure 1A; data not shown). We observed that the injection of *dsGFP* partially suppressed the ultimate level of *Defensin* induction after bacterial challenge (Figure 1A versus C), and therefore we used *dsGFP* mosquitoes as controls throughout this study. In the *dsDEF* mosquitoes, no *Defensin* mRNA of proper size was detected already at day 5 after dsRNA injection (Figure 1B and C, day 1 after bacterial challenge). Instead, a strong faster migrating signal was consistently present, accompanied by a faint signal migrating more slowly than *Defensin* mRNA. We interpret these signals as denatured and non-denatured dsRNA, respectively, as they are also exhibited by the input dsRNA (asterisks in Figure 1D). Signals corresponding to the input *dsGFP* were also detected using the *GFP* probe (bottom asterisk in Figure 1C and D), indicating that dsRNAs are stable for at least 12 days after injection in mosquitoes and therefore can provide a long-lasting inhibition of endogenous gene expression. In contrast to robust induction of *Defensin* in *dsGFP* mosquitoes (Figure 1E, days 1, 2 and 4), only traces of *Defensin* mRNA were detected in *dsDEF* mosquitoes using sensitive RT–PCR and primers corresponding to the 5′ and 3′ UTRs of the *Defensin* gene. We conclude that the injection of specific dsRNA successfully inhibits induction of *Defensin* after bacterial challenge.

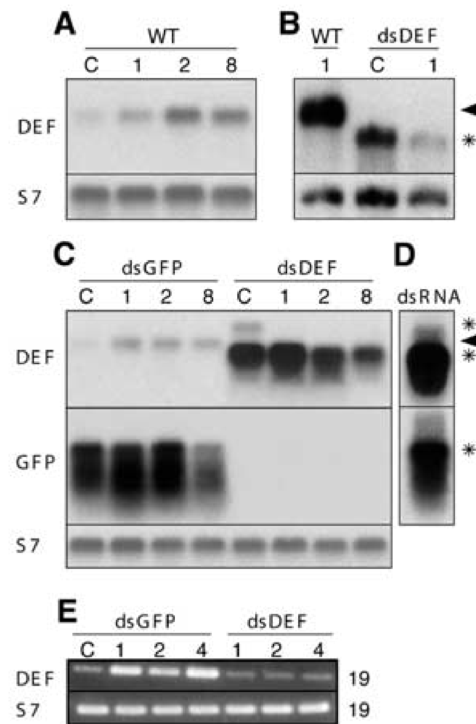

Fig. 1. RNA analysis of the *Defensin* gene knockout by dsRNA. RNA blots demonstrate that, over a period from 1 to 8 days (numbers above the images), *E. coli* challenge stably induces the expression of *Defensin* (*DEF*) mRNA (arrowheads) above the uninfected control level (C) in wild-type (WT) (**A** and **B**) and in *dsGFP*-treated, but not in *dsDEF*-treated, mosquitoes (B and **C**). In (**D**), the input *dsRNAs* match in size the signals (*DEF* and *GFP*) that are detected in dsRNA-injected mosquitoes (asterisks). (A), (C) and (D) were run on 1.2% agarose gels and (B) on an 1.4% agarose gel. The ribosomal protein *S7* transcript was used as a loading control. *GFP*, green fluorescent protein. (**E**) RT–PCR analysis of the *DEF* (19 cycles) gene expression in *dsGFP*- and *dsDEF*-treated mosquitoes before (C) and after (days 1, 2 and 4) bacterial challenge. The expression of the ribosomal protein gene *S7* (19 cycles) served as control.

The efficacy of the *Defensin* knockout was further validated at the polypeptide level by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectometry (MS) of hemolymph samples (Figure 2A). Although the *dsDEF* and *dsGFP* peptide profiles were mostly comparable, the latter showed a strong peak at 4136.2 Da, which was undetectable in the *dsDEF* sample and corresponded to singly protonated *A. gambiae* Defensin bearing three disulfide bridges (calculated mass 4136.7 Da) (Vizioli *et al.*, 2001b). This identification was confirmed by sequencing of the reduced peptide (data not shown). We also followed the *Defensin* knockout in the epithelium of the anterior midgut 24 h after infectious bloodmeal. The *dsGFP* and *dsDEF* midgut extracts again differed by a prominent Defensin peak at 4136.9 Da, which was present only in the midgut cells of *dsGFP* mosquitoes (Figure 2B). Thus, the injection of dsRNA in adult mosquitoes disrupts the expression of the targeted gene at both the RNA and polypeptide levels. Our data demonstrate that dsRNA knockout is efficient in the three different

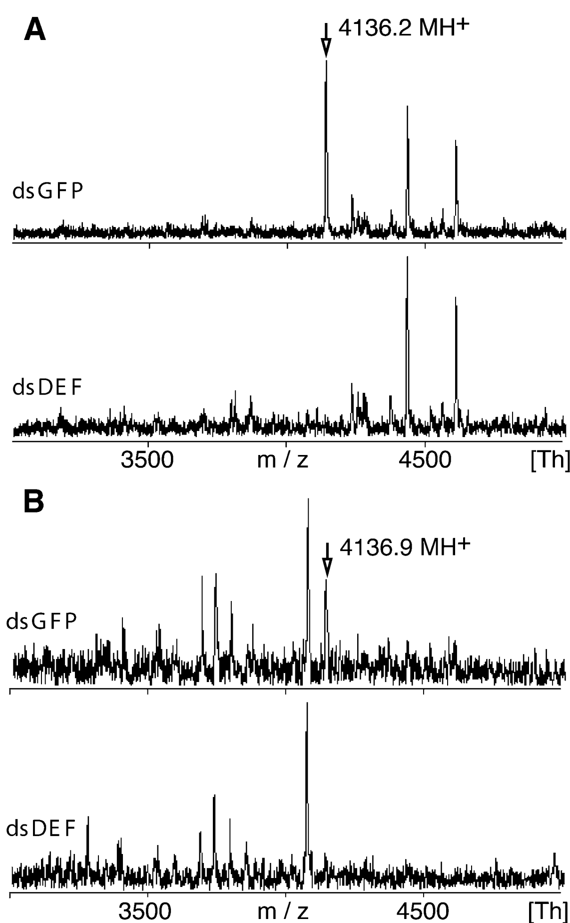

Fig. 2. MALDI-TOF MS analysis of the *Defensin* gene knockout by dsRNA. (**A**) Mosquitoes were challenged with a mixture of *E. coli* and *S. aureus*, and 36 h later the presence of Defensin peptide was detected in the hemolymph of control *dsGFP*, but not of *dsDEF*, mosquitoes. (**B**) Defensin is present in the anterior midgut 24 h after infectious bloodmeal in control *dsGFP*, but not in *dsDEF*, mosquitoes. The peaks corresponding to Defensin and their molecular weights are indicated by arrows.

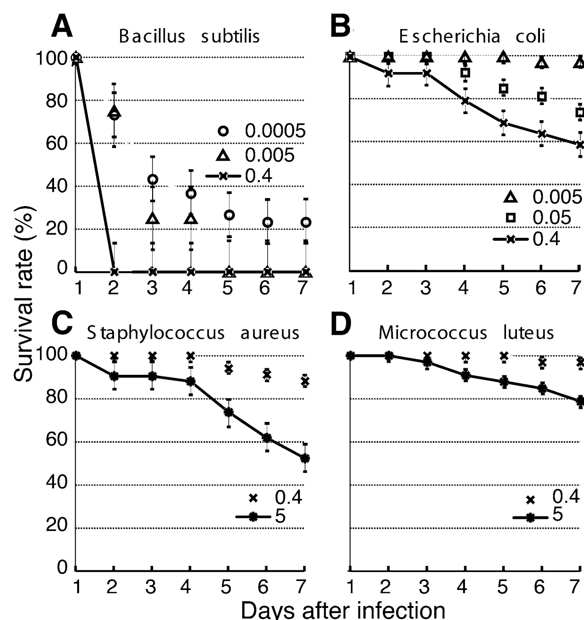

Fig. 3. Lethality of control *dsGFP*-treated mosquitoes after infection with different doses of bacteria. Survival rates (%) are presented for mosquitoes infected with (**B**) the Gram-negative bacterium *E. coli* or the Gram-positive bacteria (**A**) *B. subtilis*, (**C**) *S. aureus* and (**D**) *M. luteus*. The bacterial concentrations are expressed as optical densities (OD) of suspensions at 600 nm: OD_{600} = 0.0005 (open circles), OD_{600} = 0.005 (open triangles), OD_{600} = 0.05 (open squares), OD_{600} = 0.4 (crosses) and OD_{600} = 5 (asterisks). Each experiment was performed with 50 mosquitoes for each bacterial species, and the results shown are representative of three independent experiments. Standard errors are indicated by bars.

cell types tested (midgut, fat body and hemocytes), which originate from two distinct cell lineages: endoderm and mesoderm.

The efficacy, effectiveness and reproducibility of the knockout allowed us to determine the function and specificity of Defensin *in vivo*. Because of *in vitro* indications that Defensin is particularly potent against Gram-positive bacteria (Vizioli *et al.*, 2001b), we focused on three members of this class, *S. aureus*, *Micrococcus luteus* and *Bacillus subtilis*, as well as on a Gram-negative species, *E. coli*. We injected measured bacterial suspensions of each species into control *dsGFP A. gambiae* females and followed the survival of the mosquitoes for 7 days (Figure 3). The bacteria exhibited different degrees of pathogenicity that were independent of Gram classification. *Bacillus subtilis* rapidly killed *A. gambiae* even at concentrations as low as 85 bacteria per mosquito (Figure 3A). The next most efficient pathogen, *E. coli*, caused comparable mosquito lethality only when injected in 10-fold higher numbers; *S. aureus* was substantially less pathogenic, and *M. luteus* was the least effective (Figure 3B–D). Differential susceptibility to bacteria has also been reported in *Drosophila melanogaster*, where, surprisingly, *S. aureus*, as well as *B. subtilis*, cause rapid death, while *M. luteus* is again only weakly pathogenic (Tzou *et al.*, 2002). Comparative analysis of bacterial pathogenicity may be fruitful in pinpointing specificities of the immune response in related dipteran species.

These experiments allowed us to define the sublethal concentrations for infection with the three bacterial species other than *B. subtilis*. We injected the selected concentrations of bacteria in both *dsDEF* and control *dsGFP* females and followed their respective survival rates over a period of 7 days (Figure 4). The profiles did not differ significantly in the case of *E. coli*- and mock-injected mosquitoes. In contrast, the *dsDEF* mosquitoes were modestly susceptible to *M. luteus* (20% lethality) and highly susceptible to *S. aureus* (80% lethality). This is the first demonstration *in vivo* that a single endogenous immune peptide, Defensin, is necessary for the resistance of a mosquito to two Gram-positive bacteria and not to a Gram-negative species of bacteria. The plateau in mortality that is seen in *dsDEF* mosquitoes 5 days after infection by *M. luteus* suggests that additional immune factors or cellular processes may be implicated in the clearance of this mild pathogen.

We next examined the potential role of Defensin in the immune response of *A. gambiae* to the rodent malaria parasite, *Plasmodium berghei*. *Defensin* is constitutively expressed in the anterior midgut epithelium and is further induced by malaria

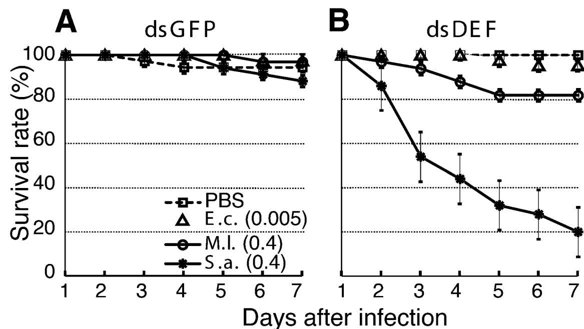

Fig. 4. *Defensin* knockdown mosquitoes are susceptible to Gram-positive, but not to Gram-negative, bacteria. The survival rates (%) of (**A**) *dsGFP*-treated mosquitoes and (**B**) *dsDEF*-treated mosquitoes after injection of PBS (open squares), *E. coli* (OD_{600} = 0.005, open triangles), *M. luteus* (OD_{600} = 0.4, open circles) or *S. aureus* (OD_{600} = 0.4, asterisks) are shown. Each experiment was performed with 50 mosquitoes for each bacterial species, and the results shown are representative of three independent experiments. Standard errors are indicated by bars.

infection (Richman *et al.*, 1997; Vizioli *et al.*, 2001b). In *Aedes aegypti*, the injection of high doses of related Defensins from a dragonfly and a fleshfly at specific time points after an infectious bloodmeal interfered with the development of the midgut stages of the parasite (Shahabuddin *et al.*, 1998). These studies prompted us to monitor parasite numbers during *P. berghei* infections in *dsGFP* and *dsDEF* mosquitoes, using as a readout the number of oocysts per midgut 10–12 days after infection. We reasoned that, if endogenous Defensin acts antiparasitically, its absence during development of the *Plasmodium* midgut stages would remove a constraint and result in higher parasite loads. We observed that the mean number of oocysts per midgut was unchanged or even slightly lower in *dsDEF* than in *dsGFP* mosquitoes (Table I). Moreover, parasite-infected *dsDEF* and *dsGFP* mosquitoes showed no significant differences in mosquito viability, ookinete/oocyst morphology or the frequency distribution of oocyst numbers. We conclude that malaria-induced endogenous *A. gambiae* Defensin does not act as a significant antiparasitic factor *in vivo*.

In conclusion, the simple and convenient dsRNA technique that we describe here efficiently disrupts gene function in distinct tissues of adult mosquitoes. As a proof of principle, we have used this method to delimit phenotypically the *in vivo* function of a single immune peptide against different types of infections, showing it to play an important role in the resistance to Gram-positive bacteria. Evidently, antimicrobial peptides that would be unaffected by *dsDEF* injection cannot substitute for Defensin. Recently, we have successfully applied dsRNA to knock out 20 additional immune genes in *A. gambiae* (data not shown), thus confirming the general validity of this method in the study of immune responses in the mosquito. With a reverse genetics method now in hand, it should be possible to conduct systematic functional genomic analysis in this major vector of human malaria.

METHODS

Mosquito colony. *Anopheles gambiae* strain G3 was reared as described previously (Richman *et al.*, 1996).

Double-stranded RNA preparation and injection in mosquitoes. dsRNAs were produced as described previously using the plasmids pLL6ds for control *dsGFP* (Levashina *et al.*, 2001) and pLL80 for *dsDEF*. pLL80 was constructed in two steps. *Defensin* cDNA of 404 bp was PCR-amplified using *dfn a* and *dfn b* primers (Richman *et al.*, 1996) and cloned into pCR2.1-TOPO (Invitrogen), resulting in pLL79. The 515 bp *Hin*dIII–*Xba*I fragment of pLL79 was then subcloned between the two T7 promoters of pLL10. Sense and antisense RNAs were synthesized using the T7 Ambion kit, annealed in water and stored as dsRNAs at –80°C until use. A nano-injector (Nanoject, Drummond) was used to introduce 69 nl of dsRNAs (1 mg/ml) in water in the thorax of CO_2-anesthetized mosquito females, which were then allowed to recover for 4 days.

RNA analysis. Total RNA was extracted from 15 mosquitoes with TRIzol Reagent (Invitrogen) and separated by electrophoresis. Two different conditions were used: to ascertain the absence of endogenous *Defensin* transcripts, the electrophoresis were performed for 6 h using 1.2% agarose gels (Figure 1B); clear signals corresponding to input dsRNAs were detected using 1.4% agarose gels and 4 h migration time (Figure 1A, C and D). Separated total RNAs were transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). Blots were hybridized sequentially with the radioactively labeled probes [Ready-To-Go DNA labeling beads (dCTP), Amersham Pharmacia Biotech]: *Defensin* (pLL79 insert), *S7* (Salazar *et al.*, 1993) and *GFP* (pLL6 insert).

For RT–PCR analysis, *Defensin*-specific primers were selected that do not overlap with the dsRNA used for the knockout: 5′-UTR primer, 5′-AAC TCC AGC CAA GCT AAA GC-3′; and 3′-UTR primer, 5′-GAA TTA AGC CTG TGT TGT AAA C-3′. Total RNA was extracted as above from whole mosquitoes at the

Table I. Survival of *P. berghei* oocysts in the *dsGFP*- and *dsDEF*-treated mosquitoes

Experiment	dsRNA	Number of midguts	Oocyst number per midgut			Mean ± SE[a] (oocysts per midgut)
			0	1–29	>30	
1	*dsGFP*	19	5	9	5	16.74 ± 3.7
	dsDEF	24	7	14	3	10.75 ± 3.1
2	*dsGFP*	13	4	9	0	2.77 ± 0.6
	dsDEF	25	7	18	0	2.48 ± 0.4

[a]SE, standard error.

indicated time points. S7-specific primers and the RT–PCR conditions were as described previously (Richman *et al.*, 1996). For amplification of both *S7* and *Defensin*, 19 PCR cycles were used.

Mass spectrometry. The hemolymph of 40 mosquitoes was collected by centrifugation of decapitated females (3000 r.p.m., 5 min) and used in 1/10 dilution in acidified water. Five dissected anterior midguts were homogenized in 0.5% trifluoracetic acid, 50% acetonitrile; and extracts were cleared by centrifugation. The samples were analyzed without further purification in a modified thin-layer preparation (Vorm *et al.*, 1994). MALDI-TOF MS analysis was performed on a Bruker Biflex (Bremen, Germany) mass spectrometer in linear positive mode using delayed extraction. Tandem MS with a nano-electrospray source mounted on a Micromass QTOF1 (Manchester, UK) mass spectrometer was used for peptide sequencing.

Bacterial challenge and mosquito survival. GFP-expressing *E. coli* OP-50 was a gift from J.J. Ewbank (INSERM, Marseille-Luminy, France). *Bacillus subtilis*, *M. luteus* and *S. aureus* were kind gifts from P. Bulet (IBMC, Strasbourg, France). Bacteria were cultured to OD_{600} = 0.4, pelleted, washed and resuspended in phosphate-buffered saline (PBS) to indicated concentrations. The number of bacteria injected was estimated by the plating of appropriate aliquots of bacterial suspension on LB plates. Mosquitoes were anesthetized with CO_2, injected into the thorax with 69 nl of the bacterial suspension or PBS for controls and allowed to recover. Mosquitoes that died within 24 h of injection were not considered in the analysis. Dead mosquitoes were daily counted and removed over a period of 7 days. The results shown here are representative of at least three independent experiments, each carried out with 50 mosquitoes per tested group.

Parasite infections and oocyst counting. Parasite infections were performed essentially as described previously (Richman *et al.*, 1997). Briefly, *P. berghei* parasites were passaged in CD1 mice, and parasitemia was determined from Giemsa-stained blood films. For each experiment, *dsGFP* and *dsDEF* mosquitoes were fed on the same infected mouse. Mosquito midguts were dissected 10–12 days later, fixed and DAPI stained. Morphology was examined, and the numbers of oocysts were counted using a UV-light fluorescent microscope (Zeiss).

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