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TREATMENT AND PROPHYLAXIS - SHORT COMMUNICATION

Double-stranded RNA reduces growth rates of the gut parasite *Crithidia mellificae*

Kleber de Sousa Pereira[1] · Niels Piot[1] · Guy Smagghe[1] · Ivan Meeus[1]

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Abstract

Parasites of managed bees can disrupt the colony success of the host, but also influence local bee-parasite dynamics, which is regarded as a threat for wild bees. Therapeutic measures have been suggested to improve the health of managed bees, for instance, exploiting the bees' RNA interference (RNAi) pathway to treat against viral pathogens. Gut trypanosomes are an important group of bee parasites in at least two common managed bee species, i.e., managed *Apis mellifera* and reared *Bombus terrestris*. In several trypanosomes, RNAi activity is present, while in other associated genes of RNAi, such as Dicer-like (DCL) and Argonaute (AGO), it is lost. Up to date, the ability to exploit the RNAi of gut trypanosomes of bees has remained unexplored. Here, we screened parasite genomes of two honey bee protozoa (*Crithidia mellificae* and *Lotmaria passim*) and two bumble bee protozoa (*Crithidia bombi* and *Crithidia expoeki*) for the presence of DCL and AGO proteins. For *C. mellificae*, we constructed a double-stranded RNA (dsRNA) targeting kinetoplastid membrane protein-11 (KMP-11) to test the RNAi potential to kill this parasite. Transfection with KMP-11 dsRNA, but also adding it to the growth medium resulted in small growth reduction of the trypanosome *C. mellificae*, thereby showing the limited potential to apply dsRNA therapeutics to control trypanosome infection in managed honey bee species. Within bumble bees, there seems to be no application potentials against *C. bombi*, as we could only retrieve non-functional DCL- and AGO-related genes within the genome of this bumble bee parasite.

Keywords dsRNA · Honey bee gut parasite · Managed bee · Parasite control · Prevention · RNAi

Introduction

Trypanosomatidae flagellates are parasites mainly discovered in insects (Podlipaev 2001). They are prevalent and abundant in different bee species and are transmitted through contact with contaminated feces inside the nest (Cisarovsky and Schmid-Hempel 2014; Schmid-Hempel and Schmid-Hempel 1993) or via shared flowers (Durrer and Schmid-Hempel 1994). The trypanosomatid *Crithidia bombi* is a very common parasite of wild bumble bees and is also found in managed bumble bees (Graystock et al. 2016; Graystock et al. 2013; Murray et al. 2013). Also, other more recent reported trypanosomes are retrieved in bumble bees, for instance, *Crithidia expoeki* can be prevalent in specific geographic areas (Gallot-Lavallée et al. 2016; Schmid-Hempel and Tognazzo 2010). *Crithidia mellificae* was initially reported as the primary trypanosome in honey bees, while more recent *Lotmaria passim* n. gen., n. sp. has been identified as being the most common (Schwarz et al. 2015). Trypanosomatids in managed bees are of importance for bee health. *Crithidia bombi* is known to have a context-dependent virulence in *Bombus terrestris*, being more harmful in starvation conditions (Brown et al. 2000), while in honey bee, trypanosome infection is correlated with winter mortality of hives (Ravoet et al. 2013).

Eradicating parasites and viruses in managed bees is important for the host itself. Furthermore, we argue that controlling diseases is important to assure that managed bees do not act as pathogen reservoirs, avoiding spill over events towards wild bees. Some known therapeutics in bees act upon RNA interference (RNAi), a natural defense mechanism of eukaryotes

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✉ Kleber de Sousa Pereira
kleber.ds.pereira@gmail.com

✉ Ivan Meeus
Ivan.Meeus@UGent.be

[1] Faculty of Bioscience Engineering, Department of Plants and Crops, Lab of Agrozoology, Ghent University, Coupure Links 653, B-9000 Ghent, Belgium

against viral infection (Cottrell and Doering 2003). This host pathway can be stimulated by administering virus-specific exogenous double-stranded RNA (dsRNA) to boost and prime immune responses. Successful examples are the treatment for Israeli acute paralysis virus (IAPV) (Chen and Evans 2012; Maori et al. 2009; Piot et al. 2015), Chinese sacbrood virus (CSBV) (Liu et al. 2010), and deformed wing virus (DWV) (Desai et al. 2012). In order to target parasites, dsRNA needs to be taken up by the parasite and the RNAi pathway must be active and able to produce small interfering RNAs (siRNAs). The reduction of the microsporidian *Nosema ceranae* with exogenous dsRNA (Paldi et al. 2010) is a successful example of parasite treatment in honey bees. Although no research data is available for trypanosomatids in bees, the potential of the RNAi has been proven for trypanosomatids infecting humans. Transfection of dsRNA resulted in successful growth reduction of *Trypanosoma brucei* (Li et al. 2008; Li and Wang 2008; Mbang-Benet et al. 2015; Ngo et al. 1998). However, since the first successful report, some contradicting results emerged. Protozoan parasites from the *Leishmania* genus seemed to lack RNAi activity (Robinson and Beverley 2003). Later, it was shown that functional RNAi in trypanosomatids was lost at least three times during speciation (Lye et al. 2010; Matveyev et al. 2017).

Here we investigated the potential of dsRNA treatment to target a cultured bee trypanosomatid parasite. We used *C. mellificae*, a trypanosome of honey bees, as a culture is available from American Type Culture Collection (ATCC) and it is phylogenetically close to multiple trypanosomatids infecting different bee species such as *C. bombi* and *L. passim* (Schwarz et al. 2015). *Crithidia mellificae* also falls within the same clade of trypanosomes presumed to have RNAi (Matveyev et al. 2017). We targeted the kinetoplastid membrane protein-11 (KMP-11) that is a highly conserved gene which plays an essential role regulating cytokinesis in the cell division process in trypanosomatids (Li and Wang 2008; Thomas et al. 2000). Furthermore, KMP-11 induces cellular and humoral immune response in infected host (Finkelsztein et al. 2015) and is reported as a virulence factor, playing an important role in the adherence and motility of trypanosomes to host cells (da Fonseca Pires et al. 2014; Finkelsztein et al. 2015; Sannigrahi et al. 2017). Therefore, it can be speculated that silencing this gene could also affect the colonization of these parasites in the gut environment of the host bee. Yet it is important to first understand if bee trypanosomes are sensitive to dsRNA treatment. We therefore first explored the available genome of four important bee trypanosomes: *C. mellificae* and *L. passim* infecting honey bees (Runckel et al. 2014) and *C. bombi* and *C. expoeki* infecting bumble bees (Schmid-Hempel et al. 2018), for the presence of functional core genes of the RNAi pathway. Next, we checked if transfection methods could introduce dsRNA (targeting *KMP-11* mRNA) into *C. mellificae* and reduce this parasite growth; for this purpose, a common electroporation and another method to optimize cell uptake using liposomes were used. Finally, to investigate spontaneous cell uptake of dsRNA and to evaluate the potential use of this molecule as a therapeutic agent, we tested if addition of dsRNA in a defined trypanosome medium influenced its proliferation.

Material and methods

Protein sequences and identification of catalytic domains within Argonaute and Dicer

A first requisite for functional RNAi is the presence of its core genes, for example, Argonaute1 (AGO1) and Dicer-like (DCL1 and DCL2). We examined their presence and looked for conserved catalytic domains in the genome of two main honey bees' protozoan parasites *C. mellificae* and *Lotmaria passim* and in two bumble bees' protozoan parasites *C. bombi* and *C. expoeki*. We used the NCBI's conserved domain finder (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). The accession numbers of the genome and protein sequences are provided in Supplementary material S1 Table 1.

The protein sequences of AGO1 and DCL1 of *Crithidia acanthocephali* (Lye et al. 2010; Matveyev et al. 2017) (the phylogenetically closed match to our focal trypanosomes with annotated protein sequences) were used to retrieve the closest orthologs. We performed tBLASTn searches against whole genome shotgun contigs databases. Nucleotide sequences were translated into protein using Expasy Bioinformatics Resource Portal (https://web.expasy.org/translate/). Adequate ORF's protein sequences were manually selected and PAZ, PIWI, and RIBOc domains were identified using the Conserved Domain Database (CCD) curated within NCBI framework (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al. 2017). When a functional domain was retrieved, protein BLAST was performed to check if the protein sequences indeed matched with AGO1, or DCL1 or DCL2 proteins of phylogenetic-related species.

We assured that our selected target is present within the genome bee trypanosomes (Supplementary material S2 Table 1). The same procedure as described above was performed to identify the *KMP-11* gene in *C. mellificae*, *C. bombi*, *C. expoeki*, and *L. passim* using blast (tBLASTn) of the annotated protein sequence from the closely related trypanosomatid *Leptomonas pyrrhocoris*. When necessary, the integrity of the imperfect ORFs was confirmed by amplifying gene problematic regions through PCR on a wild bumble bee carrying a natural infection of *Crithidia bombi*.

Phylogenetic analysis

Protein sequences obtained from our focal species (Supplementary material S1 Table 6) were aligned together with

already confirmed AGO1 and DCL sequences from other trypanosomatids (Lye et al. 2010; Matveyev et al. 2017). A same procedure was followed for the target protein KMP-11 (see protein sequences in Supplementary material S2 Table 2). To check the similarity of bee protozoans, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) was examined in 69 trypanosomatids at NCBI (https://ncbi.nlm.nih.gov) data bank. All different protein sequences (AGO1, DCL1, KMP-11, and gGAPDH) were aligned and checked manually. Amino acid sequences were aligned by Muscle with MEGA 7 software (https://www.megasoftware.net/). Phylogenetic reconstruction for each protein group were constructed by maximum likelihood (ML) method, using the best-fit model of amino acid substitution indicated by the Model Selection implemented in MEGA software. Reliability of branches was executed using 1000 bootstrap replicates.

Cell culture

The *C. mellificae* reference strain ATCC 30254 was cultured with brain heart infusion (BHI) liquid medium 37 g/l, supplemented with 3.5 ml of hemin solution (1 mg/ml in DMSO) and 2% (*v*/*v*) antibiotic mix according to Mäser et al. (2002). Parasites were grown in 10-ml tubes in an incubator at 27 °C and diluted in new medium each 3 days.

Synthesis of double-stranded RNA

Total RNA was isolated from *C. mellificae* according to RNeasy Mini Kit (Qiagen). After, the removal contaminating DNA was performed with the recombinant DNase I (rDNase I) accordingly to DNA-free kit protocol (Ambion by Life Technologies). Posteriorly, cDNA was synthesized with oligo-dT 12–18 primer and Superscript II kit on 500 ng RNA extracted (Invitrogen, Merelbeke, Belgium). The sequence of the *KMP-11* gene was retrieved from trypanosomatids on NCBI and alignment performed using BioEdit (version 7.1.9). A KMP-11 amplicon was generated after PCR amplification with the following primers: forward primer (5′-3′) GGCCACCACDYWYGARGART and reverse primer (5′-3′) TTGAAYTTVTCBGTGTGCTC, carrying a T7 promoter sequence at their 5′ part. Primers were designed using Primer3 software (Koressaar and Remm 2007). Double-stranded RNA (dsRNA) was synthesized and purified using MEGAscript RNAi kit (Life Technologies) according to the manufacturer's instructions. After purification, nuclease-free water was used to elute the dsRNA. The transcription reaction was allowed to proceed overnight. dsRNA coding for GFP was used as control, and synthesis of this dsRNA was similar as described above, starting from a plasmid (Liu et al. 2010; Piot et al. 2015).

The PCR construct of *C. mellificae* amplified with specific primers carrying a T7 promoter sequence was sent for Sanger sequencing (LGC genomics GmbH, Berlin) in both directions for characterization of this gene. BlastN analysis revealed a 100% match with *C. mellificae* KMP-11.

Double-stranded RNA delivered by electroporation

In order to test the potential of dsKMP-11 to kill or slow down growth dynamics of our test species, we mixed 5 μl of dsKMP-11 ($n = 3$) or dsGFP (control) ($n = 3$) both at 4 μg/μl with 95 μl of cells (1×10^8 cells/ml; final concentration after spinning down cells in logarithmic growth phase). Electroporation was performed as described by Robinson and Beverley (2003). After electroporation, cells were transferred immediately to fresh drug-free medium (final volume of 1 ml) at two different concentrations, 5×10^5 cells/ml and 1.5×10^6 cells/ml. Parasites were allowed to grow at 27 °C and antibiotic mix (2%, *v*/*v*) was added to the fresh medium 5 h later. In total, we performed two independent experiments (each with three repetitions). Cells were counted on an improved Neubauer's bright line chamber with a × 40 objective lens (Will-Wetzlar, Wetzlar, Germany) over a period of 4 days. Parasite growth rate was determined for each day, and growth rate at day x is the ratio of the amount cells counted at day x over day $x - 1$.

Double-stranded RNA delivered by liposomes

For the transfection with liposomes, 5 μl of gene-specific dsKMP-11 or dsGFP both at 4 μg/μl was diluted separately in BHI medium reaching a final volume of 100 μl each. Twenty microliters of Escort IV reagent (Sigma) at 1 μg/μl was added to 80 μl BHI medium. Then, both dsRNA and liposomes were mixed and incubated at room temperature for 45 min allowing the complexes to form. Next, the mixture 1:1 of dsRNA with liposomes was gently added to 800 μl of BHI medium (final volume of 1 ml) containing cells ($6–7 \times 10^5$ cells/ml) and incubated for 18 h at 27 °C. Day after, cells were allowed to grow in regular growth medium ($5–7 \times 10^5$ cells/ml) at 27 °C. Three biological repetitions were performed for each treatment. Parasite growth was checked daily with a hemocytometer and growth rate was determined as mentioned above.

Delivery of double-stranded RNA via in vitro growth medium

DsRNA needs to be taken up by the parasite in order to use dsRNA-based therapeutics against trypanosomatid parasites in bees. To test this, we performed an in vitro experiment via soaking mixing 5 μl of dsKMP-11 or dsGFP (control) both at 4 μg/μl with 995 μl of regular growth medium containing cells. We performed two independent experiments with parasite densities between 2 and 3×10^5 cells/ml, and two

additional experiments with densities between 5 and 7×10^5 cells/ml. All four independent experiments (separated in time) had three repetitions. Parasite growth was checked in four time points (0–3 days) with a hemocytometer and growth rate was determined daily for 3 days. We only used growth rate of the first day for statistical analysis, as here we could assure that the cells were still in logarithmic growth phase.

Statistical analyses

Growth rate day 1 was log transformed as this improved its normality. The data from transfection via liposome was subjected to normal Student's *t* test since only single experiment with three repetitions was performed.

For the electroporation and soaking experiments, we used linear models in R. We had two independent electroporation experiments. We included "experiment" as a factor since different cell densities were used. The treatment with dsKMP-11 is the factor of interest. For the soaking study, there were four independent soaking experiments with two different cell densities. The difference in cell density had a clear effect on the growth rate in these assays. Hence, we performed separate statistics on the low- and high-density experiments. Again, "experiment" and "treatment" were two level factors. Omitting the interaction between both factors did not improve the models' AIC scores and had no influence on the results. Figures were plotted in Sigmaplot software.

Results and discussion

The RNAi pathway has been lost several times in different lineages from the subfamily Leishmaniinae (Robinson and Beverley 2003; Lye et al. 2010; Matveyev et al. 2017), which includes the main bee trypanosomatids from the genus *Crithidia* and *Lotmaria*. Therefore, preceding our experimental procedures, we screened if the core proteins of the RNAi pathway are present. We retrieved the major participants Dicer-like and Argonaute in the genomes of *C. mellificae*, *C. expoeki*, and *L. passim*, revealing a potential efficient RNAi system (Supplementary material S1 Tables 1 and 2). The retrieved nucleotide sequences within the genome of *C. bombi* which matched with DCL and AGO were incomplete because of frameshift mutations and the introduction of stop codons. These mutations were confirmed with Sanger sequencing of a wild strain (Supplementary material S1 Figs. 1 and 2) and the protein coding sequences do not contain the characteristic functional domains. The retrieved protein sequences for *C. mellificae*, *C. expoeki*, and *L. passim* had high similarity with other published sequences of AGO1 and DCL1 (Supplementary material S1 Fig. 3). All domains Paz and Piwi for AGO and RIBOc domain in DCL were conserved for *C. mellificae*, *C. expoeki*, and *L. passim* (Supplementary material S1 Tables 3 and 4). These domains are described to be present within kinetoplastids with functional RNAi (Matveyev et al. 2017). Therefore, we conclude that a functional RNAi can be present in *C. mellificae*, *C. expoeki*, and *L. passim*, while it is probably lost in *C. bombi*.

For the in vitro tests, we focused on if dsRNA can be used as treatment against on the honey bee parasite *C. mellificae*. To the best of our knowledge, it is the first time tested in a bee protozoan. We targeted KMP-11 mRNA, an essential protein in all kinetoplastids, by triggering the RNAi machinery of the parasite. First, we used two transfection methods to assure the dsKMP-11 enters the parasite cells. We noticed that the parasites treated with dsKMP-11 had a lower growth rate in both experiments; in both methods, this effect was only visible as a trend (with electroporation: $F_{\text{treatment}} = 5.03$; $P = 0.055$; with liposome: $P = 0.1$; Fig. 1). The non-significant parasite reduction after electroporation can be attributed to an inconsistent efficiency (Hasenkamp et al. 2012), ranging from 20 to 50% in other trypanosomatids (Gomaa et al. 2017). Different factors contribute to the electroporation success, with dsRNA being different from double-stranded DNA, but also the parasite age, damage (Barros et al. 2017), and density (Hasenkamp et al. 2012) are important factors to consider.

The transfection experiment showed the potential of the technique, but probably uptake was not efficient enough; this could become an important hurdle in order to use dsRNA as a potential drug. We tested if addition of dsKMP-11 directly in the parasite's culture medium can influence growth of *C. mellificae*. Here, the parasite's growth with dsKMP-11 treatment was significantly lower in the experiment when cells were cultured at a high density ($F_{\text{treatment}} = 7.50$; $P = 0.03$; Fig. 2a). In contrast, when cells were grown at a low density, then no significant effects were noticed ($F_{\text{treatment}} = 0.88$; $P = 0.38$; Fig. 2b). The latter is a consequence of the higher variability in growth rate in these experiments (also in the control treatment) (Fig. 2b).

Although we noticed in both transfection and soaking experiments a recovery of *C. mellificae* growth 2 days post-treatment (Supplementary material S3), the knocking down of KMP-11 had a prolonged effect in *Trypanosoma brucei* (Li and Wang 2008). We see a combination of possible reasons for this. Firstly, the rapid growth rate of *C. mellificae* noticed in our experimental setup. Secondly, due to the high proliferation rate, cells quickly entered the stationary growth phase, allowing the slower doubling parasites to also reach their saturating densities. Thirdly, the dsRNA might lose stability in our tested medium which could reduce its availability in the experiments. Finally, also in *T. brucei*, dsKMP-11 treatment does not block mitosis completely (Li and Wang 2008).

The concentration of dsKMP-11 used in soaking experiment had significant, yet a minor effect on the parasite growth

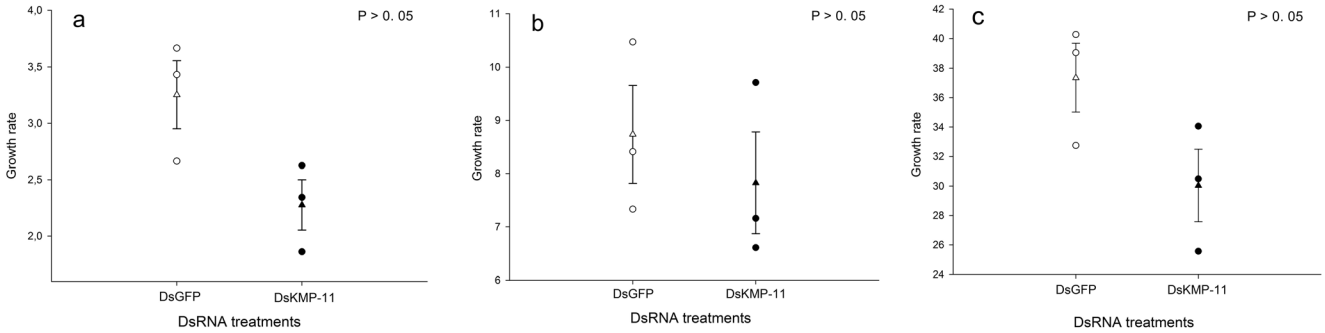

Fig. 1 Growth rate of parasites (mean ± SE) after 1 day of treatment with dsGFP and dsKMP-11 by two transfection methods. Electroporation experiments with exponential growth phase of 1.5×10^6 cells/ml (**a**) and with 5×10^5 cells/ml (**b**) (three biological repetitions each). **c** Experiment showing exponential growth phase of 5–7 × 10^5 cells/ml after inoculation of dsRNA treatments and liposome (three biological repetitions). Standard errors indicated with error bars; means of parasites treated with dsGFP (white triangle) and dsKMP-11 (black triangle) are represented. Each symbol (white and black circle) represents the observed data points of growth rate after day 1

rate. However, we still argue that the dsRNA treatment can possibly result in an application. Overall, we showed that adding dsKMP-11 in the culture medium of *C. mellificae* induced a mean decrease of growth rate of 3.9 ± 1.0. Treatment based on dsRNA reduced the parasite of *N. ceranae* more than 3-fold in honey bee gut (Paldi et al. 2010). These positive results of in vivo tests underline the stability and potential of dsRNA molecules to target diseases in bees after oral administration. We argue that targeting bee trypanosomes with dsRNA is a valid option to pursuit. KMP-11 is a relevant target, also used in therapeutic programs aiming antibody and vaccine production to protect humans against protozoans (Finkelsztein et al. 2015; Fuertes et al. 2001). In our setup, we showed that soaking *C. mellificae* with exogenous dsRNA to silence KMP-11 resulted in growth reduction of this protozoan under optimal culture media and high density. Nonetheless, we can expect that in bee gut conditions, the growth of trypanosomes is less favorable, due to lower food availability and presence of competitors.

The dsRNA technology holds the potential to target multiple related parasites that are RNAi-proficient. Indeed, a dsRNA molecule in a conserved region of a trypanosome-specific protein can easily target multiple related parasites. Furthermore, bee trypanosomes are phylogenetically related (see Supplementary material S1 Fig. 4, showing the gGAPDH housekeeping gene phylogenetic tree for multiple trypanosomes). Thus, the KMP-11 gene, also conserved among trypanosomes (Supplementary material S2 Fig. 1), is an interesting target. On the other hand, dsKMP-11 will probably not target all bee trypanosomes as shown for *C. bombi* where this parasite does not encode functional RNAi proteins. Although we take the first step towards in vivo application, caution is needed since dsRNA uptake and delivery are key aspects to be considered in order to have a good in vivo response. Once these hurdles are dealt with, trypanosomes are ideal target parasites, particularly as they are exclusively present in the gut while delivering dsRNA to other bee parasites that infect different tissues is less feasible.

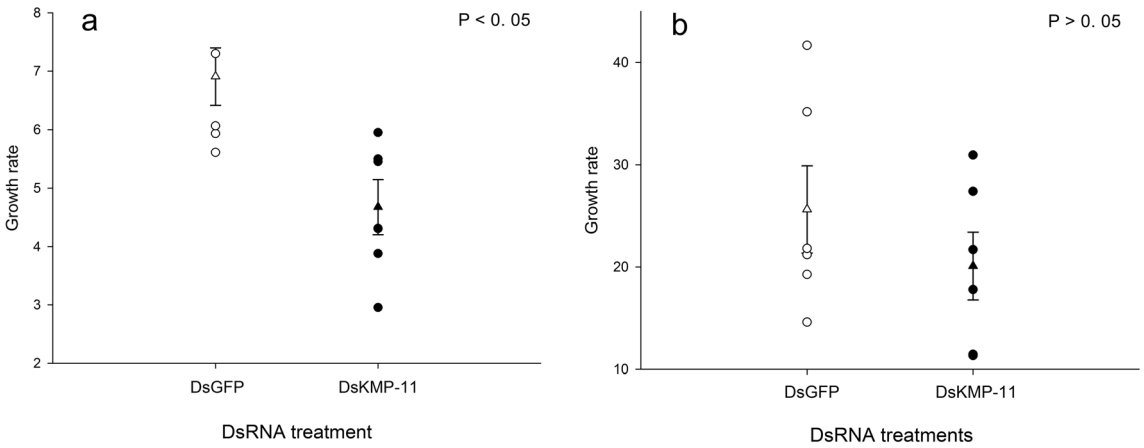

Fig. 2 Growth rate (mean ± SE) post 1 day treated with dsGFP and dsKMP-11 by soaking. **a** A combination of two experiments with a higher parasite density of 5–7 × 10^5 cells/ml and **b** a combination of two experiments with a lower parasite density between 2 and 3 × 10^5 cells/ml. Each experiment was performed with three biological repetitions. Standard errors indicated with error bars; means of parasites treated with dsGFP (white triangle) and dsKMP-11 (black triangle) are represented. Each symbol (white and black circle) represents the observed data points of growth rate after day 1

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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