The shutting down of the insulin pathway: a developmental window for Wolbachia load and feminization

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Using the isopod Armadillidium vulgare as a case study, we review the significance of the "bacterial dosage model", which connects the expression of the extended phenotype to the rise of the Wolbachia load. In isopods, the Insulin-like Androgenic Gland hormone (IAG) induces male differentiation: Wolbachia feminizes males through insulin resistance, presumably through defunct insulin receptors. This should prevent an autocrine development of the androgenic glands so that females differentiate instead: feminization should translate as IAG silencing and increased Wolbachia load in the same developmental window. In line with the autocrine model, uninfected males expressed IAG from the first larval stage on, long before the androgenic gland primordia begin to differentiate, and exponentially throughout development. In contrast in infected males, expression fully stopped at stage 4 (juvenile), when male differentiation begins. This co-occurred with the only significant rise in the Wolbachia load throughout the life-stages. Concurrently, the raw expression of the bacterial Secretion Systems co-increased, but they were not over-expressed relative to the number of bacteria. The isopod model leads to formulate the "bacterial dosage model" throughout extended phenotypes as the conjunction between bacterial load as the mode of action, timing of multiplication (pre/post-zygotic), and site of action (soma vs. germen).

Wolbachia are likely the most widespread endosymbionts on Earth, infecting arthropods such as insects, mites, spiders and crustaceans, but also parasitic nematodes. These maternally transmitted endosymbionts proliferate by manipulating the reproduction of their host through four main extended phenotypes (male-killing, parthenogenesis, Cytoplasmic Incompatibility (CI), feminization) or by developing an obligate interaction with their partner. The expression of the extended phenotypes was repeatedly related to the bacterial load. In a seminal study on Nasonia sp. and CI, a "bacterial dosage model" was proposed in which a cross is effective when the oocyte harbours equal or greater numbers of Wolbachia than the spermatocytes. Basically, in incompatible crosses, CI prevents normal mitosis at the first embryonic division or during embryogenesis, leading to embryonic mortality. In Nasonia sp. however, the bidirectional CI is complicated by the haplodiploid determination system. Incompatible crosses result in the elimination of paternal chromosomes and therefore yield only haploid males, as if the eggs had not been fertilized. In compatible crosses, antibiotic curing of Wolbachia in mothers gradually decreases the bacterial load in the oocytes, shifting the sex ratio in successive broods from all-female to all-male. As incompatibility translates the non-rescue from a toxin secreted by the bacteria in the sperm, this "bacterial dosage model" reflects a pre-zygotic action of Wolbachia in male cysts. Similar inferences were further proposed from correlating CI levels and cyst infection frequency. In Drosophila melanogaster, wMel infects only 8% of testes cysts and displays a low CI effect. When transinfected in D. simulans, the CI effect shifts to high, while cyst infection reaches 80%. Similar correlations were made in different wRi-infected Drosophila hosts.
Furthermore, quantitative analyses in several species such as D. simulans, Aedes albopictus or the isopod Porcellio dilatatus also correlated the strength of unidirectional CI with the Wolbachia load\cite{11,12}.

The “bacterial dosage model” can be expanded to the other extended phenotypes. In D. bifasciata harbouring a male-killing strain, high temperatures reduce the bacterial load in mothers to a threshold below which Wolbachia is no longer able to kill males, thus producing Wolbachia-bearing males\cite{13}. In Hymolimnas bolina, preventing bacterial proliferation in mothers postpones the death of the sons to a larval stage, upon Wolbachia recovery\cite{14}.

In Muscidifurax uniraptor, the Wolbachia-induced production of diploid females through parthenogenesis is altered in a dose-dependent manner by rifampicin treatments of the mothers, that result in a decrease of the bacterial load: the higher the dose of rifampicin, the higher the proportion of haploid males\cite{15}. Again, and in both cases, the proper execution of the extended phenotypes is conditioned by the bacterial load at a pre-zygotic level, as depleting the generation N-1 hampers their expression. As for effectors, there is no telling whether they are produced in the zygote, or stock-piled in the maternal generation, mirroring the paternal toxin in CI.

In contrast, mutualistic and feminizing strains mostly act at the post-zygotic level, continuously throughout the embryonic and/or larval development, or even throughout the life of their host. In the mutualistic relationship with Brugia malayi\cite{16}, microfilariae and L1 to L3 larval stages in the mosquito host are poorly infected, whereas the Wolbachia load dramatically increases in L3 larvae upon transmission to a mammalian host\cite{17}. According to Landmann et al.\cite{18}, antibiotic treatments disrupt especially L3/L4 larvae development, probably as a consequence of inhibited bacterial division\cite{19}. These observations suggest that Wolbachia is involved in the development of late larval stages, possibly participating to a kind of metabolic complementation scheme\cite{20}. Wolbachia similarly conditions embryo and adult survival, although this was not connected with a heightened bacterial load\cite{21,22}. In the bed bug Cimex lectularius, Wolbachia provides the host with B vitamins, leading to a nutritional mutualism\cite{23,24}. Wolbachia-cured embryos and larvae suffer growth defects and display a lower adult emergence rate. Bacterial titres increase dramatically between the first and the fifth instar stages, correlatively to the essential role of Wolbachia for embryonic development\cite{25}.

Very much in contrast with the other reproductive extended phenotypes, feminization displays a diversity of mechanisms, well reflected in the comparison of three Wolbachia strains. All systems generate phenotypic functional females, but with different genotypic identities: “de-sexualized” Z0 (Eurema mandarina, known first as E. hecabe yellow-type), genetic females XX (Zygnyidia pullula), genetic males ZZ (Armadillidium vulgare)\cite{26,27}.

If the prevalence of females increases, so does the transmission of Wolbachia: this is the case in E. mandarina (~ 100% females), A. vulgare (~ 80% females), but not in Z. pullula (~ 50% females). Indeed in Z. pullula, infected genetic males (X0) become intersexes with more or less damaged ovaries: they are mostly dead-ends as progenies are very seldom observed\cite{28,29}. Here, feminization swaps the sex-specific profile of the genomic imprinting patterns from male to female, ahead of sex realisation cascades and differentiation processes, possibly targeting a master control of the latter\cite{30}. That a threshold load of bacteria is necessary for feminization is expected from the observation that 1% intersexes retain testes instead of ovaries, and a male imprinting profile instead of a female one, in conjunction with a lower bacterial load\cite{28,29}. In contrast, feminization in E. mandarina acts as a two-step mechanism, during sex determination and differentiation. Wolbachia-infected females are Z0 instead of WZ, following the exclusion of the maternal sex chromosomes during or after meiosis, so that each new generation is Z0\cite{31,32}. Whereas Wolbachia-cured Z0 individuals are not viable, in infected ones Wolbachia compensates for the absence of the W chromosome somewhere along the sex realisation cascade, ultimately imposing the female doublesex splicing variant\cite{33}. That the male splicing variant could or should be expressed instead is revealed by curing individuals during the larval development, which results in the differentiation of intersexes expressing both splicing variants, in conjunction with decreased bacterial loads\cite{34}.

In our model A. vulgare, feminization results in sex reversal and appears to be purely post-zygotic. Here, sex differentiation is orchestrated by a masculinising hormone produced by the androgenic glands: the Insulin-like Androgenic Gland hormone (IAG)\cite{35,36}. It supersedes the sex-determination processes. Indeed, sex differentiation at the juvenile stage 4 can be surgically reversed for yet another couple of molts: WZ females are fully masculinised by the grafting of an androgenic gland\cite{37}, while ZZ males spontaneously become fertile females upon the ablation of the primordia of the androgenic glands\cite{38}. Therefore, female is the default sex. In ZZ males, the androgenic gland primordium begin to proliferate at stage 4\cite{39} and become fully visible as tissues at stage 6\cite{40}.

Wolbachia infection shunts this process, without altering the number of chromosomes\cite{41,42} (~ 80% of a progeny turn into functional phenotypic females and occasional intersexes, while the remaining ~ 20% males result from both splicing variants, in conjunction with decreased bacterial loads\cite{43}.

In infected, phenotypic females, the androgenic glands never become visible, but can be revealed upon partial curing by temperature: in each gonad, the third androgenic gland primordium re-activates, leading to re-masculinisation\cite{44}. This shows that they retain a functional male determination and differentiation pathway, and that it is the latter that would be dampened somehow by Wolbachia. In intersexes, feminization is delayed, so that female-like intersexes retain a single vestigial androgenic gland per gonad, while male-like intersexes display fully developed and even hypertrophied androgenic glands; however, if cut, their copulatory pleopods regenerate in a female form\cite{45}. In fact, all infected individuals become impervious to the masculinising effect of the IAG, be it synthesized by their own glands or by grafted ones: in other words, feminization is a form of insulin resistance. According to Juchault and Legrand\cite{46}, it is the receptor of the IAG (the insulin-like hormone) that would be defunct. This hints to an autocrine mechanism in the differentiation of the androgenic glands, where shunting these receptors would prevent their development. Hence, the question that arises is when does IAG refractoriness occur and is it related to increased Wolbachia densities?

Here, we report that genetic males expressed the IAG during the larval stages even before the androgenic gland primordia begin to differentiate, and in accordance with their ZZ genotype, regardless of the presence or absence of Wolbachia. However, in infected individuals, the IAG gene expression fully stopped at the juvenile stage 4, 5 or 6 at the latest, i.e. the time-window for male differentiation. This was matched by a bacterial-load increase at stage 4. Together with the Wolbachia depletion experiments of Rigaud et al.\cite{47}, our results show that,
in this model again, the bacterial load is instrumental to the execution of the extended phenotype. Concurrently, the raw expression of the bacterial Secretion Systems (T1SS and T4SS) co-increased at stage 4, but they were not over-expressed relative to the number of bacteria. We discuss the possibility that effectors themselves could be constitutively expressed. The execution of any extended phenotype could therefore reflect the conjunction of bacterial load, timing of multiplication, and site of multiplication or invasion, adding further dimensions to the bacterial dosage model.

**Results and discussion**

To compare the infection load of animals of different developmental stages (Fig. 1), we quantified the number of *Wolbachia* genomes relative to the number of host cells using the ratio of two single-copy genes: the *wsp* gene for *Wolbachia* and the *IAG* gene for the host[^42]. The bacterial load was quite constant during the larval stages (1–3; *p* values > 0.05), during the juvenile stages and in young adults (stages 4–8; *p* values > 0.05). In between, the bacterial load rose sharply (4.9-fold between stages 3 and 4; *p* value = 0.027). In comparison with stage 8, the 1-year-old adults displayed a 4-fold increase, which was however not significant (*p* value = 0.208), with a high variation between individuals. Here, inter-individual variability was likely inflated by fluctuations of the *Wolbachia* load during the reproductive cycle, for example when *Wolbachia* accumulates in the ovaries: more oocytes get infected as ovaries mature[^48]. Overall, the only effective increase in the bacterial load coincided with the time of sexual differentiation: in isopods, the gonads start to differentiate at stage 4, then at stage 5 the external sex characters begin to appear in males[^36].

In *A. vulgare*, feminization is expected to disrupt the autocrine development of the androgenic glands: it implies that in normal males, the IAG should be expressed early in their precursor cells, even before the glands differentiate at stage 6[^36]. Precursor cells were described by Juchault[^35] at stages 4 and 5, when male gonads differentiate, in the suspensory tracts where the glands will develop. He also predicted that the precursors already exist in the undifferentiated gonads in the larval stages. Congruently, in the males of the *Wolbachia*-free lineage, the IAG gene was expressed from birth on and exponentially throughout development, including in the larval stages (1–3) and in differentiating juveniles (4–6) (Fig. 2A). Females on the other hand are not expected to express the IAG gene: this was however reported in some decapod models, in connection with a supplementary role in metabolism (e.g.[^45]). Since we sampled the larval stages as pools that contained genetic females as well, we cannot verify whether larval females expressed the IAG initially. But even if they did, and at a significant rate, it would not matter for differentiation: Suzuki[^44] demonstrated that larval females are refractory to IAG and cannot be reversed by the graft of an androgenic gland. As concerns juvenile females, they did express the IAG gene (31/36 females from stages 5 to 8), but at an extremely low and constant rate (2.10^{-4} as a mean), that contrasted with the exponential expression in males. This resulted in an increasing gap between females and males, from a 79-fold difference at stage 5 to a 488-fold difference at stage 8 (Fig. 2A). In any case, such a weak expression was not sufficient to trigger a biological response in male differentiation. Overall, in line with the literature, we infer that the crux of male differentiation relates to the expression level of the IAG gene from stage 4 to stage 6, allowing the differentiation of the gonads and the androgenic glands.

In the *Wolbachia*-infected lineage, it is in this developmental window that we observed an endocrine disruption. The IAG gene was initially expressed in the larval pools, but at a rate that matched that of stage 4 uninfected males (Fig. 2B), and that was even higher than in the uninfected lineage where the prevalence of males is 50% (Mann–Whitney test; stage 1: mean = 0.006 vs. 0.001, *p* value = 0.028; stage 2: mean = 0.006 vs. 0.004, *p* value = 0.18, ns; stage 3: mean = 0.005 vs. 0.001, *p* value = 0.002). Therefore, the larvae seemed to express the IAG gene according to their genotype (100% genetic males), not their future phenotype which depends on the

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[^31]: Female
[^35]: Male
[^42]: The number of bacteria was estimated by qPCR using the *wsp* gene, normalised by the single copy nuclear gene *Av-IAG* from the host. Pools of undifferentiated larvae were sampled after birth (stage 1), one and two weeks after birth (stages 2 and 3, respectively). For the next developmental steps, *Wolbachia*-infected animals were sampled individually: undifferentiated juveniles (stage 4), and phenotypic females (stages 5–8) until adulthood (AF for adult females).

[^45]: Female
[^46]: Male
Wolbachia infection status (~20% future males lacking Wolbachia, ~80% future females with Wolbachia). This expression of the IAG gene in infected animals was however in conjunction with a low Wolbachia load in larvae (Fig. 1). In contrast, from stage 4 on, the expression of the IAG mRNA was related to the Wolbachia infection status (Fig. 2B). Uninfected juveniles all expressed the IAG mRNA, whereas most Wolbachia-positive juveniles did not, despite their ZZ genotype. Some juveniles expressed the IAG gene while being infected by Wolbachia, but their prevalence dropped to zero over two stages: from 31% (5/16) at stage 4, 14% (2/14) at stage 5, to none in the later stages (stage 6: 0/11; stage 7: 0/18; stage 8: 0/14). They can correspond to future phenotypic females, Wolbachia-infected individuals being insensitive to IAG, or to intersexes. However, the prevalence of intersexes in this lineage is much lower than this: over the last seven years only 10 intersexes were harvested among 2,532 individuals (0.39%) of 69 litters from our rearing. These individuals were thus probably future functional females wherein the IAG gene expression was about to get silenced in the presence of Wolbachia.

In either case, the time-window for a successful feminization in A. vulgare can be narrowed down to stages 4 and 5, which co-occurred with the only significant rise in the Wolbachia load. Earlier, even regular females are refractory to IAG, so that Wolbachia preventing the expression of the IAG would be superfluous. Later, once the precursors of the androgenic glands degenerate (during stage 6), females are terminally differentiated, so that they do not produce IAG anyway. In the fertile, female-like intersexes, male-differentiation is probably shunted in this window as well: only the first pair of androgenic gland primordia begins to develop and aborts. In contrast, in the sterile, male-like intersexes, feminization is delayed beyond these stages so that they have time to develop three functional pairs of androgenic glands before they become refractory to IAG. Imperfect feminization could result from an insufficient Wolbachia load, leading to a belated extinction of the IAG gene, allowing partial male differentiation. Indeed, partial curing by temperature during larval development generates an excess of male-like intersexes. Moreover, Rigaud et al. inferred from bioassays that female-like intersexes contain less bacteria than phenotypic females in their somatic tissues, whereas the bacterial load in ovaries is similar. While infecting ovaries is important for vertical transmission, disabling the receptors of the IAG for feminization is a body-wide symptom. A question is whether the heightened Wolbachia load serves to match such a broad target, or, closer to the model of Juchault and Legrand, to reach and disable discrete endocrine centres that control the functionality...
of all IAG receptors. In other words, within the "bacterial dosage model", we consider the localisation of the heightened dose of Wolbachia in terms of site of action for the extended phenotype.

In this, Wolbachia strains that act at the post-zygotic level share common traits that contrast with those acting at the pre-zygotic level. For the latter, the site of expression of the extended phenotype matches the site of vertical transmission: it is a common target, to be resolved in the N-1 generation. That location matters could therefore not be a part of the initial model of Breeuwer and Werren in Cl. The post-zygotic acting Wolbachia on the other hand, must target a somatic niche in addition to the germinal niche, the expression of the extended phenotype in the soma promoting indirectly vertical transmission in the germin. Similar to what we observed in A. vulgare, the execution of the extended phenotype is connected to a heightened bacterial load in the developmental stages. In B. malayi, this increase is observed in the somatic lateral chords where Wolbachia would complement host metabolism, even before the ovaries get infected in L4 females. In C. lectularius, Wolbachia is concentrated in the bacteriome plus ovaries: the increase of the bacterial load in individuals along larval development probably reflects the expansion of Wolbachia in these niches, following an early colonization in embryogenesis. Slightly closer to our model, E. mandarina harbours a feminizing Wolbachia strain; still, the differences with the isopod system are of a magnitude, since sex determination in insects is "cell-autonomous" and is enforced by differentiation, cell by cell. In the absence of a hormonal coordination, Wolbachia needs to act earlier than the larval stage, during embryogenesis, so that a homogenous sex background emerges in each cell. In this, Wolbachia may need to be represented in all cells. Its action (possibly its global colonization) must be sustained further, all along development, or intersexes are obtained. Intersexes express both the male and the female splicing variants of doublesex, and are mosaics of sexual characters; maybe it is a question of bacterial load at the scale of the cells, that would result in a mosaic expression of the splicing variants. In A. vulgare, we rather suspect that Wolbachia targets specific endocrine cells.

The "bacterial dosage model" entails a further dimension, the mode of action of Wolbachia to execute the extended phenotype. Intrinsic to this concept is that it is mediated through, so to speak, strength in numbers. Mechanistically, this should translate as an increased delivery of bacterial substances in the host cytosol, namely metabolites or effectors, transferred through secretion systems. Stage-specific expressions or over-expressions could complement this process, but this is not quite the picture drawn in the literature. So far, only putative effectors are known, except for CI, cifA and cifB causing mitotic failures in the first stages of embryonic development. They are expressed in succession within the window of heightened Wolbachia load in sperm cysts, and cifA, which doubles as a rescuer, in adult females. They are however also expressed throughout embryonic development, by the bacteria inherited from the mother. cifA itself is expressed only at very low levels in the early stages: rescue could stem from stock-piled maternal CifA instead, mirroring the paternal origin of the toxin. As regards post-zygotic acting Wolbachia, global transcriptomic studies in filarial models record L3/L4-specific products, differentially expressed products or enriched GO-terms, but these data are not normalized against the Wolbachia load. As for secretion systems, T4SS is expressed continuously throughout the life stages, Normalizing its expression or that of its transcription factors (WbmX1 and WbmX2) against the Wolbachia load reveals an under-expression only in microfilariae. In A. vulgare, T1SS and T4SS were identified in the ongoing sequencing project of wVuIC (Liu et al., unpublished results). The T1SS is encoded by three genes (tolC, hlyB, hlyD) scattered in the genome, and the T4SS by the virB3-virB6 and the virB8-virD4 operons. Hence, we followed the expression of these secretion systems throughout that of the tolC, virB3 and virB8 genes along the developmental stages of A. vulgare. While the raw expression of the three genes increased from stage 4 on, the normalized expression remained constant from stage 1 to stage 8, and in adult females (Fig. 3A-C). Like the bacterial load, the gene expression was highly variable in adults, likely due to the different reproductive states of the females. The permanent expression of the genes encoding these secretion systems in A. vulgare and in nematode models may result from their involvement in symbiotic processes that go beyond the execution of the extended phenotype. All the same, for these systems and for effectors, a regulation of expression through bacterial numbers may prove more plastic and adaptive than a regulation wired in transcription dynamics, that demands synchronising with pinpoint accuracy not only with the host's cell, but with its life cycle.

Conclusion
In unifying our perception of the "bacterial dosage model" throughout the extended phenotypes of Wolbachia, we have formulated this concept through the notions of timing of expression (pre/post-zygotic acting strains), site of action, and mode of action. In A. vulgare, analysing in situ the fine distribution of Wolbachia within the tissues during the different stages of development will allow to discriminate the site of action of feminization from the site of vertical transmission. It will determine whether site colonization stems from distributing bacteria between daughter cells during embryogenesis, or entails a posterior migration of bacteria between organs. Indeed, in B. malayi, the larval gonads are free of Wolbachia until they are secondarily colonised by the bacteria from the lateral chords. At the interface between site and mode of action, Juchault and Legrand predict that Wolbachia invades the endocrine centres that control the activity of the receptors of the IAG (i.e. insulin receptors): grafting tissues containing these centres to male-like intersexes restores insulin sensitivity and therefore male differentiation. In other words, through Wolbachia, we are in search of an unforeseen level of control in the insulin pathway: a canonical switch in insulin sensitivity, the shutting of which leads to insulin resistance.

Material and methods
Biological material. Armadillidium vulgare (Malacostraca, Isopoda) individuals used in this study come from two lineages: a Wolbachia-free lineage originating from Nice (France) and a Wolbachia-infected lineage originating from Celles-sur-Belle (France), wherein females harbour the feminizing wVuIC strain. These lineages have been stably maintained in the laboratory since 1967 and 1991, respectively. All animals were reared in a controlled environment with a 12:12 h light/dark cycle.
under laboratory conditions in boxes containing wet compost and food ad libitum (dried lime tree leaves and fresh slices of carrots), at 20 °C, under the natural photoperiod. In both lineages, several crosses were followed up in order to harvest animals at each post-embryonic developmental stage36. Larval stages were sampled in pools of 20–40 newly hatched individuals for stage 1, pools of 10–20 individuals one week later for stage 2 and two weeks after birth for stage 3. Juvenile animals were sampled individually and corresponding stages (4–8) were determined according to their size36. Finally, one-year-old males and females were sampled, well after sexual differentiation. All samples were stored immediately after harvesting in liquid nitrogen. The experiment was performed on at least 5–6 biological replicates for pooled samples, 9–13 individual juveniles and 5–6 biological replicates per adult.

**RNA and DNA extraction.** Whole animals or animal pools were homogenized using a Vibra Cell 75,185 sonicator (amplitude of 35%). RNA and DNA were extracted from each *A. vulgare* sample using the Qiagen AllPrep DNA/RNA Mini Kit according to the manufacturer’s recommendations. RNA and DNA quantity was assessed by NanoDrop spectrophotometry.

**Quantification of the *Wolbachia* load by quantitative PCR (qPCR).** *Wolbachia* density was determined in each DNA sample previously extracted from young *A. vulgare* at different post-embryonic stages by
qPCR amplification of the Wolbachia surface protein (wsp) gene from Wolbachia and the IAG gene from A. vulgare.

The qPCR reactions were performed using Applied Biosystems SYBR Green master mixes (5 µL Sybergreen 5X, 0.5 µL of each primer (10 µM; Supplementary Table S1), about 20 ng of DNA and sterile water up to 10 µL). The reactions were performed with a technical replicate on a LightCycler 480 System (Roche), using the following program: 95 °C for 10 min followed by 45 cycles of (95 °C for 10 s, 60 °C for 10 s, 72 °C for 20 s). Melting curves were established (65–97 °C) to check the specificity of the PCR products. The bacterial density was calculated in copy number of the bacterial genome normalized to the copy number of the host genome using Ct values and the LightCycler 480 Software. Statistically significant differences between groups were analysed with a Kruskal test followed by Dunn’s post-hoc tests implemented in the R package PMCMR using a p value = 0.05 and the Holm’s correction for multiple comparisons. The differential expression of the IAG gene between the Wolbachia-infected and the Wolbachia-free lineages was checked with a Mann–Whitney test, as specified in the text.

Quantification of gene expression by reverse transcription (RT)-qPCR. The expression of the IAG gene was evaluated during A. vulgare development. T1SS and T4SS expression were also measured by quantification of the expression of tolC, virB3 and virB8 genes, using RNA of the same samples for which the bacterial load has been determined. RT were carried out on 500 ng of total RNA using random primers and the SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) following the supplier’s instructions. qPCR reactions were performed as already described using 2.5 µL cDNA and primers given in Supplementary Table S1. The IAG and secretion system gene expression levels were analysed relatively to the RbL8 and wsp gene expression, respectively, using Ct values and the LightCycler 480 Software.Statistically significant differences between groups were analysed as described above.

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PG., J.B., B.H., S.G. and B.S. conceived and designed the study. B.H., S.G., C.D., M.R. and J.L. performed the experiments. B.H., J.B., PG. analysed and interpreted the data. J.B. wrote the paper together with PG. and B.H. PG., J.B., B.H., S.G. and B.S. revised the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare no competing interests

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