The effect of *Plasmodium vivax* infection on *SOCS* gene expression in *Anopheles dirus* (Diptera: Culicidae)

Kittiwattanawong, K.[1], Ponlawat, A.[2], Boonrotpong, S.[1], Nanakorn, N.[3], Kongchouy, N.[4], Moonmake, S.[5] and Pengsakul, T.[3*]

[1]Department of Biology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
[2]Vector Biology and Control Section, Department of Entomology, Armed Forces Research Institute of Medical Sciences, Ratchathewi, Bangkok 10400, Thailand
[3]Faculty of Medical Technology, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
[4]Department of Mathematics and Statistics, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand
[5]The Office of Prevention and Control 12 Songkhla, Department of Disease Control, Songkhla 90110, Thailand
[*]Corresponding author e-mail: theerakamol.p@psu.ac.th
Received 19 August 2019; received in revised form 16 December 2019; accepted 17 December 2019

Abstract. The *Anopheles dirus* mosquito is a primary malaria vector that transmits many species of *Plasmodium* parasites in Thailand and is widely spread across its geographic area. In the current study, the levels of expression of the suppressor of cytokine signaling (*SOCS*) gene in *An. dirus* mosquitoes infected with *P. vivax* were examined. The level of the gene's expression determined by mRNA extraction in *An. dirus* females (n=2,400) was studied at different times (0, 12, 24, 36, and 48 h after feeding), with different types of blood feeding (non-feeding, parasite-negative blood feeding, parasite-positive blood feeding) and in different parts of the body of mosquito samples (thorax and abdomen). The datasets were analyzed based on their relative expression ratio by the $2^{-\Delta\Delta C_T}$ method and were tested for significant differences with ANOVA. The results showed that the *An. dirus SOCS* gene was stimulated in the abdomen 12 h and 24 h after blood feeding about three times more highly than in unfed females, with the difference being significant. At 24 h after *P. vivax*-infected blood feeding, the *SOCS* gene in the abdomen was expressed more highly than 24 h after parasite-negative blood feeding and expression was almost 36 times higher than in the control group who were not fed blood. However, in the thorax at all times after feeding and non-feeding, there was no expression of the *SOCS* gene. Therefore, the *SOCS* gene in *An. dirus* was most highly expressed 24 h post-feeding with a *P. vivax*-infected bloodmeal, which indicates that the *SOCS* gene in the major malaria vector in Thailand plays an important role in its immune system and its response to *P. vivax* infection.

INTRODUCTION

Malaria outbreaks are an important health problem in Thailand with the disease being transmitted by the *Anopheles* mosquito. There are three primary vectors comprising *Anopheles dirus*, *Anopheles minimus*, and *Anopheles maculatus* which are highly potent vectors in the transmission of *Plasmodium* parasites in Thailand (Jongwutiwes & Thisayakon, 2006). Moreover, in Thailand, *Plasmodium falciparum* and *Plasmodium vivax* are the major malarial parasites and

have been the cause of many deaths over a long period of time (Jongwutiwes & Thisayakon, 2006; Niamnuy, 2012). Many previous studies have focused on anti-malarial drug resistance, the treatment of malaria in humans, and genetic variation in the parasites (Jongwutiwes & Thisayakon, 2006; Wells *et al.*, 2010) and molecular techniques have also been used to investigate malaria in both mosquitoes and human hosts. For example, multiplex PCR was used to detect different species of malaria by Padley *et al.* (2003) and Johnston *et al.* (2006)

extended nested PCR to a multiplex PCR technique, which produced high quality and precise detection and identification of malaria species.

Nowadays, many studies investigate the biology of vectors and parasites, and in particular, the malarial parasite genome, with the aim of discovering new drugs by focusing on genetic variation in a number of genes which contribute to drug resistance in malaria parasites (Jovel *et al.*, 2011). The immune system in mosquitoes has been studied with reference to factors including the immunodeficiency signaling pathway (*IMD*), Janus kinase (*JAK*), the signal transducer and activator of transcription (*STAT*) pathway, and genes related to the mosquito's immune system, such as, the nitric oxide synthase (*NOS*) gene, the suppressor of cytokine signaling (*SOCS*) gene, the heme peroxidase 15 (*HPX15*) gene, and the thioester-containing protein 1 (*TEP1*) gene. The *IMD* pathway and the *JAK-STAT* pathway are both highly conserved in dipterans and very important in insect development, insect homeostasis, and the activation of some genes involved in the mosquito's immune response against parasite infection, such as, the *NOS* gene which produces nitric oxide as a device to eliminate invasive parasites (Waterhouse *et al.*, 2007; Bahia *et al.*, 2011) and the *TEP1* gene which produces thioester-containing protein to stimulate lysis of the ookinetes (Vlachou & Kafatos, 2005; Smith *et al.*, 2014; Liew *et al.*, 2017) (Fig. 7).

SOCS proteins have been studied in *Drosophila* and it was shown that the *SOCS36E* protein was responsible for development in eggs and pupae (Callus & Mathey-Prevot, 2002). *Drosophila melanogaster* was studied in relation to the *STAT* pathway in the presence of bacterial infections and it was shown that the activity of the *STAT* pathway increases during infection (Agaisse & Perrimon, 2004; Arbouzova & Zeidler, 2006). In mosquitoes, it was shown that the *SOCS* proteins and their role in the immune system were stimulated when *Aedes aegypti* was infected by the dengue virus, and that the infected mosquitoes expressed higher levels of *SOCS* genes than uninfected mosquitoes (Souza-Neto *et al.*, 2009). *SOCS* gene expression in *An. culicifacies* has also been studied in relation to *P. berghei* and the results showed that the *SOCS* gene is an important gene in the immune system of *Anopheles* mosquitoes and is highly expressed in infected mosquitoes. *An. Culicifacies*, which is a major malaria vector in India, which transmits both *P. falciparum* and *P. Vivax*, was found to produce an immune response to fight malaria (Dhawan *et al.*, 2015).

Other studies have suggested new ways to control and prevent malaria in the future with some genes being found to accidentally contribute to parasite invasion in mosquitoes. The *HPX15* gene is an evolutionarily conserved gene in *Anopheles* mosquitoes, which occurs in the genome of 19 *Anopheles* species worldwide, and it plays an important role in developmental stages, as well as in ecdysis and mating. However, *HPX15* is suspected of being responsible for helping parasite invasion in mosquitoes. The gene is highly expressed in a mosquito's digestive tract when it feeds in order to decrease the immune system response and create a so-called, low immunity zone. When the mosquito is fed infected blood, this can accidentally support *Plasmodium* invasion into the midgut lumen. (Kajla *et al.*, 2015; Kajla *et al.*, 2017).

In this study, the level of *SOCS* gene expression was investigated at different times, on different types of blood meals, and in different parts of the body of female *Anopheles* mosquitoes after feeding on blood containing *P. vivax*. Genetic control represents the frontier in malaria control and this study will contribute to the use of gene-based techniques in combatting outbreaks of malaria in Thailand as well as in other parts of the world.

MATERIALS AND METHODS

Ethics Statement
The protocol for rearing mosquitoes in this study was certified by the Institutional Animal Care and Use Committee, Prince of Songkla University, under reference number *2561-10-045*. The method for collecting and

using the infected blood was certified by The Ethics Committee for Research in Human Subjects, Department of Disease Control, and was compliant with *ICH-GCP*, under reference number *61037*.

Mosquitoes and parasites

An. dirus (laboratory strain) were reared at 26 ± 2°C and 80% relative humidity (Gary & Foster, 2001; Spitzen & Takken, 2005) in the Department of Biology, Faculty of Science, Prince of Songkla University. The *P. Vivax*-infected blood fed to the mosquitoes was provided by a patient with hyperparasitemia (2-5%) and a high number of gametocytes (Hanscheid, 1999). This experiment used blood from a single patient to ensure that all the blood used was affected by an equal level of parasitemia. The patient whose blood was used permitted the collection of blood and agreed via a consent form certificated by The Ethics Committee for Research in Human Subjects, Department of Disease Control, conforming to *ICH-GCP*. Parasite-negative blood was provided by the blood bank, Faculty of Medical Technology, Prince of Songkla University All the blood was preserved in citrate-phosphate-dextrose (CPD) at a blood:CPD ratio of 6:1. CPD is a suitable medium to aid cell survival and inhibit the blood-clotting process (Moore *et al.*, 1991).

Experimental design

2,400 female mosquitoes were divided into three pools of 800 mosquitoes each and the three pools were fed (or not fed) different blood as the non-feeding, parasite negative blood-feeding, and parasite positive blood-feeding groups. Specimens were collected at five times (0, 12, 24, 36, 48 h after feeding) in pooled-sample replications (five pools at each time point, n = 30/pool).

Feeding, collection, preservation, and dissection of mosquitoes

Blood was provided to the mosquitoes using an artificial feeding technique (Fig. 1). The female *Anopheles* mosquitoes were fed on blood for 30 minutes and maintained at 26 ± 2°C in a dark room. Soaked cotton balls containing 6% glucose solution were placed in the mosquito cups (Gary & Foster, 2001; Spitzen & Takken, 2005). The pooled samples at 0 h after feeding were collected immediately, with further pooled samples being collected 12, 24, 36, 48 h after feeding. The collection and dissection processes were identical at different time points. The mosquitoes (n=30 per pool) were collected using a mouth aspirator and dissected to separate the thorax and abdomen using sterile forceps with 10x PBS buffer to maintain the mosquito's cells. The dissected mosquitoes were inspected by stereomicroscopy with an aseptic technique (Fig. 2). The thorax and abdomen of the mosquitoes were separately preserved in cryogenic vials in RNA preservative solution (RNAlater™ Stabilization Solution, Thermo Fisher Scientific Inc., USA) and stored at -80°C to maintain the RNA.

RNA/DNA extraction and cDNA synthesis

Total RNA and total DNA were isolated using an All Prep DNA/RNA/Protein Mini kit (Qiagen, USA) following the manufacturer's instructions. The total RNA was treated with DNase I (DNase I, RNase-free, Thermo Fisher Scientific Inc., USA) to eliminate all the genomic DNA in the total RNA extraction process. The RNA product was stored at -80°C for conversion to cDNA and the DNA product was stored at -20°C. The yield and quality of the total RNA were determined by a spectrophotometer (DS-11 Spectro-photometer, DeNovix, USA), and were diluted to achieve equal concentrations in all the samples. First-Strand cDNA was synthesized from the total RNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA) following the manufacturer's protocol, in a reaction volume of 20 µl.

Sequencing of *SOCS* mRNA gene and identification of the malaria parasite

The cDNA was amplified by gene-specific primer pairs, SOCSF2 5' CAG GTC GAC TTY ATY CAC TG 3', SOCSR2 5' CGR TAG TGG TAC TCT TTC AGG 3' employing the following steps: initial denaturation at 95°C for 5 minutes, 35 cycles of 10 seconds at 94°C, 30 seconds at 48°C, and 60 seconds
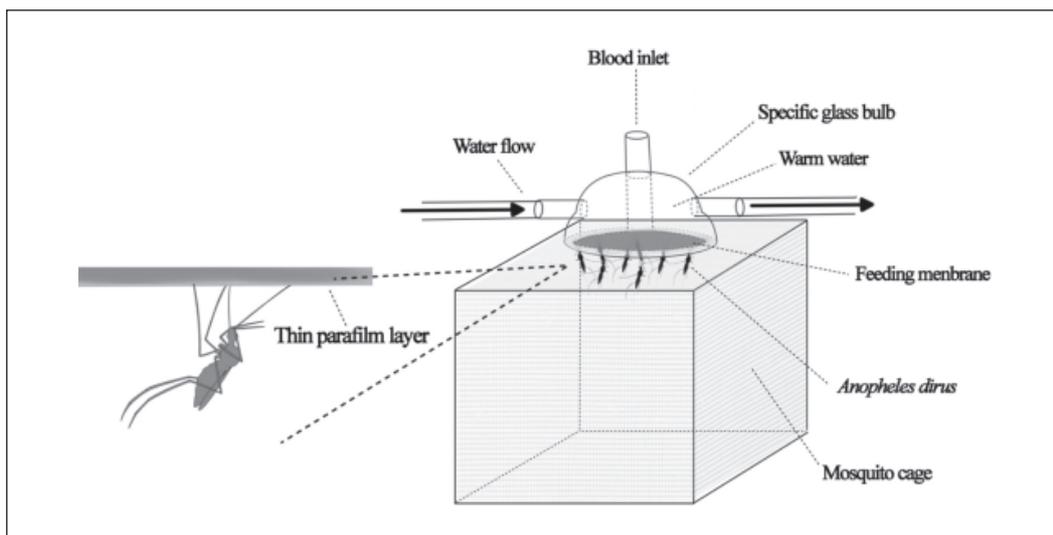
Figure 1. *An. dirus* was fed via artificial feeding. This method mimicked natural mosquito feeding using a thin parafilm layer similar to a mammal's skin, with warm (37 ± 1°C) water flowing through a plastic tube to maintain the temperature, circulated by a water pump (SONIC AP1600, China) in a water bath (DAIHAN scientific, Korea). The mosquito cage size was 30 x 30 x 30 cm. The diameter of the glass blub was 10 cm. The blood was dispensed 2 ml at a time from the glass bulb.
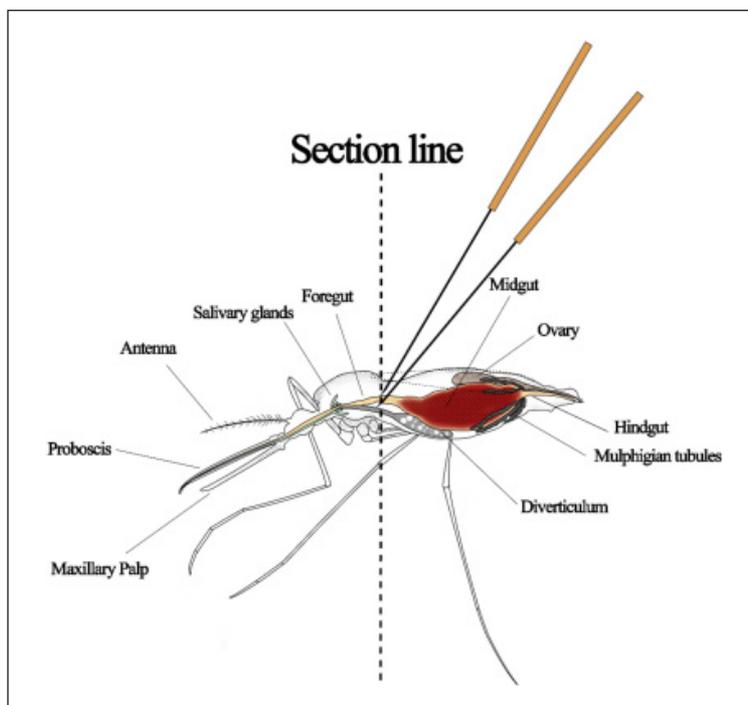
Figure 2. The *An. dirus* were dissected to separate the thorax (consisting of the thorax, wings, and legs) from the abdomen.

at 72°C, and a final extension at 72°C for 10 minutes for sequencing (Liew *et al.*, 2018). The sequences were confirmed by submission to NCBI for BLAST. The total DNA was used to confirm *P. vivax* infection in the infected blood which was fed to the mosquitoes. The identification of *P. vivax* followed the world standard protocol based on nested PCR using gel electrophoresis (Johnston *et al.*, 2006).

Quantitative Real time PCR of *SOCS* gene and gene expression statistical analysis

Quantitative real time PCR (qPCR) was conducted with a Roche Light Cycler 480 (Rocher, Germany) using SYBR Green (SYBR GreenER^{TM} qPCR SuperMix Universal, Invitrogen, USA) (Derveaux *et al.*, 2010) in a reaction volume of 20 µl. All the reactions were performed in two technical replications per pool using five pools for analysis. The *S7* gene was used as an internal loading control (Salazar *et al.*, 1993; Eisenberg & Levanon, 2013; Liew *et al.*, 2017). The primer pairs, *S7F* 5' GGC GAT CAT CAT CTA CGT 3', *S7R* 5' GTA GCT GCT GCA AAC TTC GG 3' were used to amplify the *S7* gene with an expected size of 132 bp and 108.4% PCR efficiency (Dixit *et al.*, 2007; Derveaux *et al.*, 2010; Liew *et al.*, 2017), and *SOCS-FW* pairs 5' CGT CGT ACG TCG TAT TGC TC 3', *SOCS-RV* 5' CGG AAG TAC AAT CGG TCG TT 3' were used for amplification of the *SOCS* gene with an expected size of 241 bp and 96.88% PCR amplification efficiency (Fig. 3) (Kubista *et al.*, 2006; Dhawan *et al.*, 2015; Svec *et al.*, 2015). The real-time PCR cycle followed the following steps: initial denaturation at 95°C for 5 minutes, 45 cycles of 10 seconds at 94°C, 20 seconds at 58°C, and 30 seconds at 72°C. A fluorescence reading was taken at 72°C after each cycle. The melting curve was measured from 65 to 95°C to confirm the qPCR products and the products were also checked by gel electrophoresis (Dhawan *et al.*, 2015). The cycle threshold was calculated by original software on the Roche Light Cycler 480 and all the reactions were exported to calculate the relative expression ratio by the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001).

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) to detect statistically significant differences and Dunnett's, family error rate comparisons were made with the non-feeding control group (depending on the treatment). The data were analyzed, and graphs constructed using Minitab® version 16.2.1 (USA). All data are expressed herein as mean ± SD and differences were considered to be statistically significance if the p-value for each experiment was less than 0.05.

RESULTS

The level of gene expression in different blood conditions

The result of the statistical analysis of the qPCR of the pooled sample replications (5 pools for each timepoint, n = 30/pool) based on the mean ± SD revealed that at the inception of the experiment 0 h after feeding there was no significant difference in the level of *SOCS* mRNA expression in either the abdomen or the thorax of the pools of experimental mosquitoes (fed negative blood or positive blood) or of the control group (non-feeding). However, 12 h after blood-feeding, the level of *SOCS* mRNA in the abdomen of the negative blood-feeding pool and the parasite positive blood-feeding pool were respectively 2.96 ± 1.48 and 3.81 ± 1.61 times (F = 6.480, p ≤ 0.05) higher than in the non-feeding at 12 h after inception (control group). Further, 24 h after blood-feeding, the level of *SOCS* mRNA in the abdomen of the negative blood-feeding pool and the parasite positive blood-feeding pool were respectively 3.64 ± 1.82 and 35.96 ± 3.14 times (F = 430.96, p ≤ 0.001) higher than in the non-feeding at 24 h after inception (control group). Nevertheless, after 36 and 48 h in the abdomen and at all times (after 12, 24, 36 and 48 h) in the thorax, the level of *SOCS* mRNA in both the experimental groups showed no significant (p < 0.05) change in expression compared with the control group (Fig. 4).
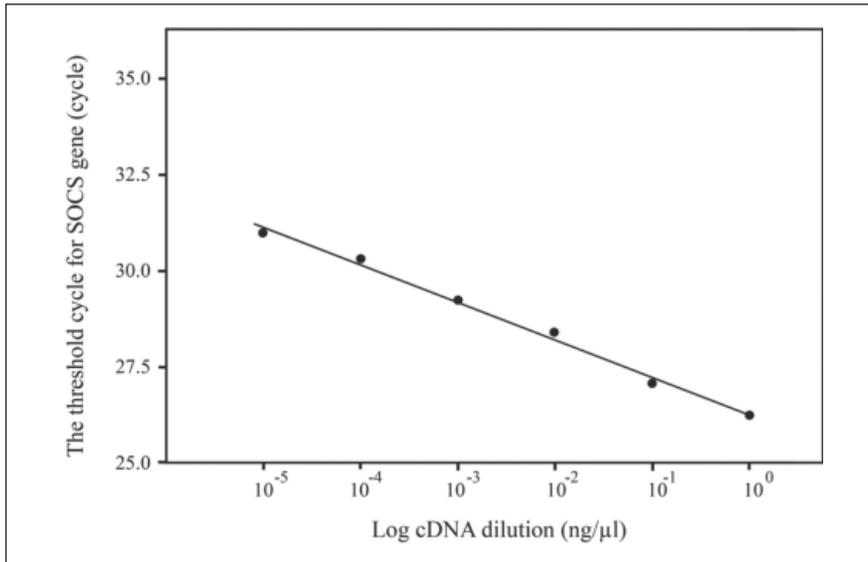
Figure 3. The *SOCS* primer amplification efficiency was calculated from the threshold cycle for the *SOCS* gene with Log cDNA dilution (ng/µl) to construct a standard curve following the equation: % Amplification efficiency (E) = $(10^{-(1/slope)} -1) \times 100$ (Kubista *et al.*, 2006; Svec *et al.*, 2015).
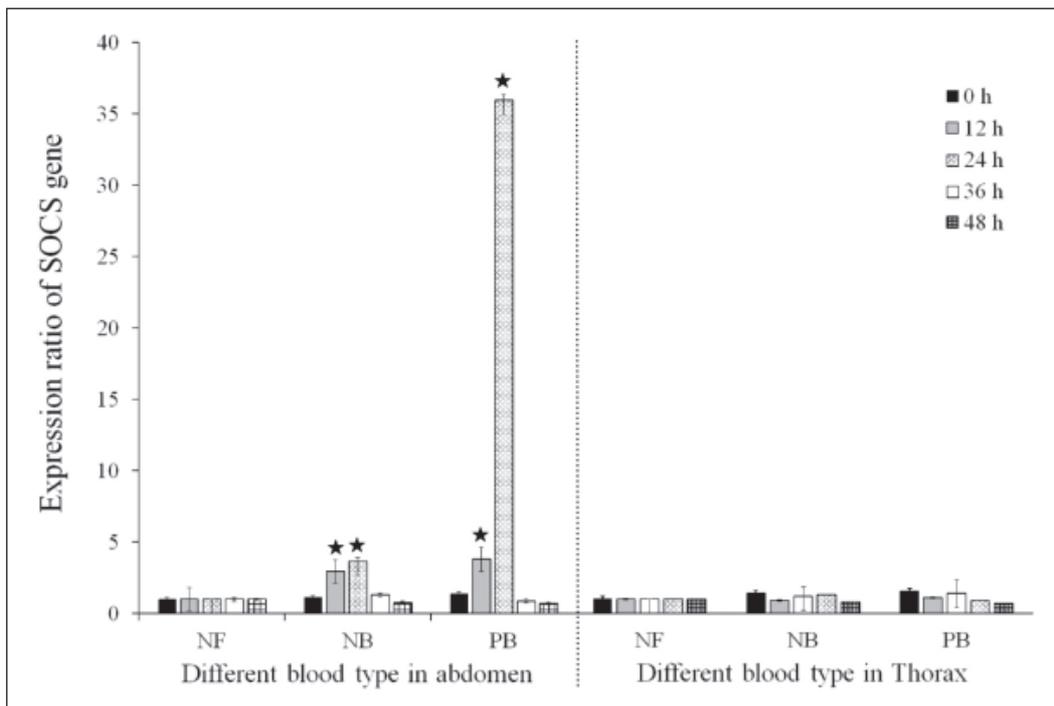
Figure 4. The expression of the *SOCS* gene after feeding on different blood types; NF = non-feeding (control), NB = negative blood-feeding, and PB = parasite positive blood-feeding. Expression ratio of the *SOCS* gene in an *Anopheles* mosquito's abdomen and thorax at different times (0, 12, 24, 36, and 48 h after feeding) as established by qPCR amplification. The process used the ribosomal protein *S7* gene as an internal loading control. The results are mean ± SD of pooled sample replications (5 pools, n = 30/pool).

★ Statistically significant difference ($p < 0.05$).

The level of gene expression based on different time conditions

The result of the statistical analysis of the qPCR of the pooled sample replications (5 pools for each time point, n = 30/pool) revealed that the levels of *SOCS* mRNA in the abdomen of the negative blood-feeding group at 12 h and 24 h after blood-feeding were, respectively 3.27 ± 1.78 and 4.99 ± 2.06 times (F = 11.830, $p \leq 0.001$) higher than in the negative blood-feeding pooled samples 0 h after feeding (control group). Further, after 12 h and 24 h, the levels of *SOCS* mRNA in the abdomen of the parasite-positive blood-feeding group were 3.75 ± 1.85 and 25.22 ± 3.79 times (F = 148.8, $p \leq 0.001$) higher than in the positive blood-feeding pooled samples 0 hour after feeding (control group). However, at other times there were no significant differences in the levels of *SOCS* mRNA expression in the abdomen and thorax of the control group, the negative-blood feeding group, or the parasite-positive blood-feeding group (Fig. 5).
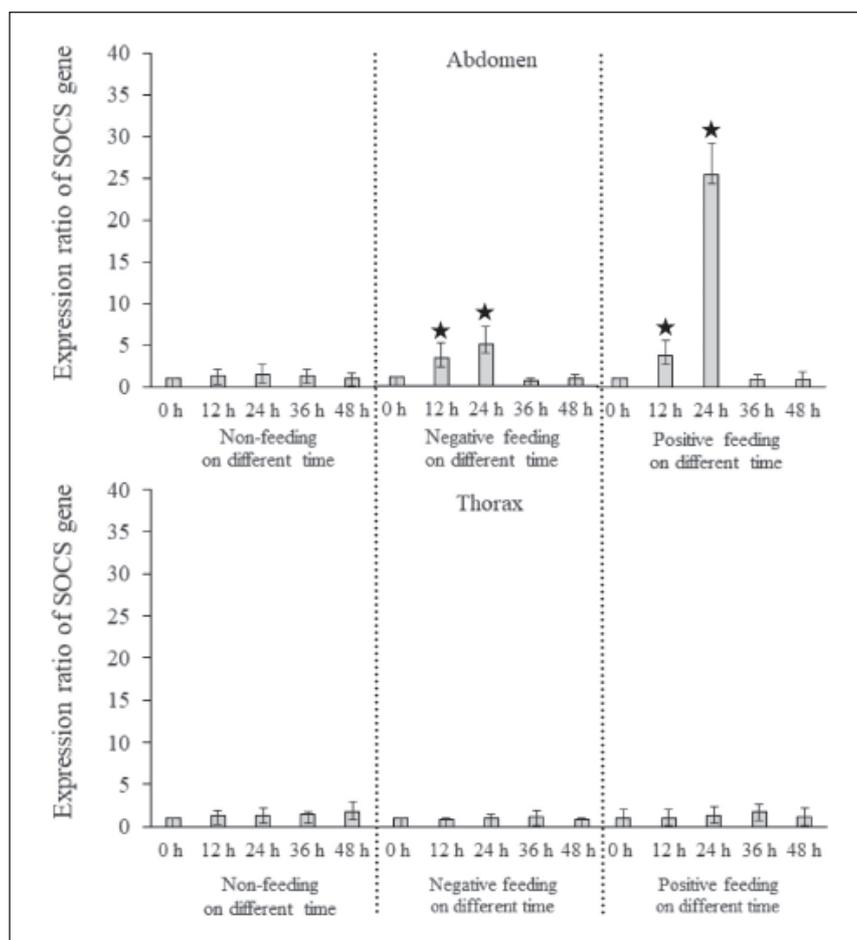
Figure 5. The expression of the *SOCS* gene after feeding on different blood types; NB = negative blood-feeding, and PB = parasite positive blood-feeding, based on different numbers of hours after feeding. The expression ratio of the *SOCS* gene in an *Anopheles* mosquito's abdomen and thorax on different blood treatments (non-feeding, negative blood-feeding or parasite positive blood-feeding compared with 0 h after feeding) at each time investigated based on qPCR amplification. The process used the ribosomal protein *S7* gene as an internal loading control. The results are mean ± SD of pooled sample replications (5 pools, n = 30/pool).
★ Statistically significant difference (p < 0.05).

The level of gene expression in different parts of the body

The result of the statistical analysis of the qPCR of the pooled sample replications (5 pools for each time point, n = 30/pool) based on the mean ± SD, revealed that the level of *SOCS* mRNA in the abdomen of the negative and parasite-positive blood feeding groups 12 h after feeding were respectively 3.80 ± 2.10 and 3.62 ± 1.71 times (F = 13.74, p ≤ 0.05) higher than in the thorax 12 h after feeding (control group). Further, after 24 h, in the abdomen of the negative and parasite positive blood-feeding groups, the levels of expression of *SOCS* mRNA were respectively 3.88 ± 1.41 and 20.73 ± 2.14 times (F =10.64, p ≤ 0.05) higher than in the thorax 24 h after feeding (control group). However, at other times and based on the different blood feeding regimes, the level of expression of *SOCS* mRNA in the abdomen and thorax showed no significant differences (Fig. 6).

DISCUSSION

In this study, it was found that the levels of *SOCS* gene mRNA expression were at higher levels in the abdomen both 12 and 24 h after feeding with blood infected with *P. vivax* when compared with the thorax and other blood feeding regimes, and that the differences in level were statistically significant. Therefore, the result can be summarized as showing that the blood infected with *P. vivax* had an effect on the expression of the *SOCS* gene in *An. dirus* causing it to be more highly expressed. Moreover, at both 12 and 24 h after feeding with parasite-negative blood, the levels of
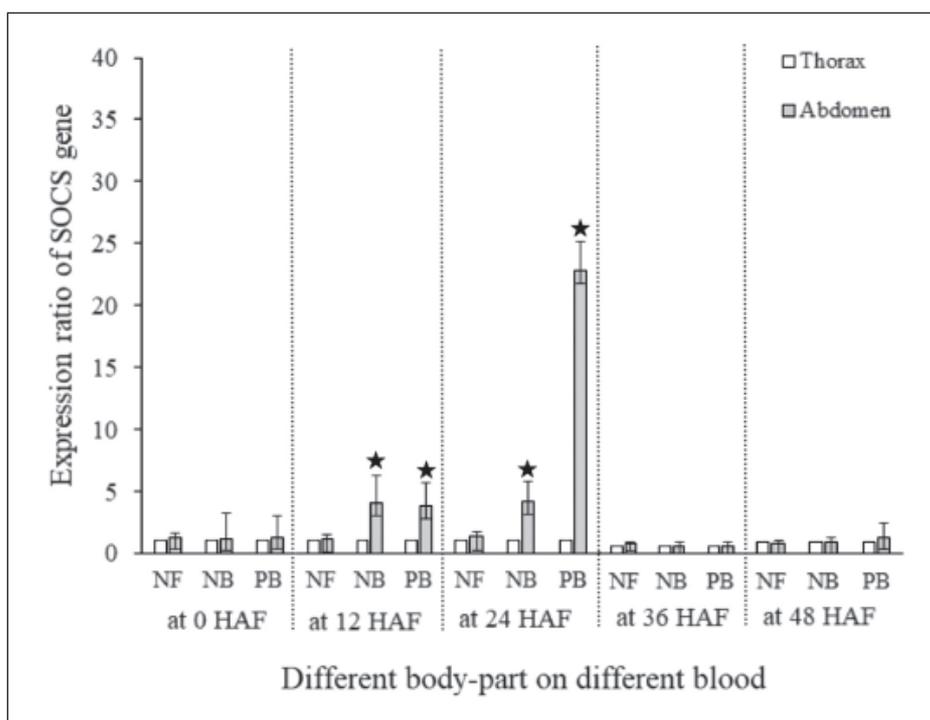
Figure 6. The expression of the *SOCS* gene after feeding on different parts of the body, compared on different blood treatments; NF = non-feeding, NB = negative blood-feeding, and PB = parasite positive blood-feeding.The expression ratio of the *SOCS* gene in an *Anopheles* mosquito's thorax and abdomen on different blood treatments investigated by qPCR amplification. The process used the ribosomal protein *S7* gene as an internal loading control. The results are mean ± SD of pooled sample replications (5 pools, n = 30/pool). HAF = hour after feeding.
★ Statistically significant difference (p <0.05).

SOCS gene mRNA expression in the abdomen were also elevated. This result indicates that blood was able to induce the *SOCS* gene, even though it was not infected with *P. vivax*. This result is important in understanding the relationship between the *Anopheles* mosquito and the *Plasmodium* parasite.

Previous research has identified the *Anopheles* mosquito as a successful malaria vector with the ability to transmit the *Plasmodium* parasite to other hosts. The life cycle of *Plasmodium* in the mosquito depends on a female *Anopheles* mosquito ingesting gametocytes which then develop into erythrocytes. Within 20-24 h, gametocytes develop into ookinetes and invade the basal lamina where they transform into the oocyst stage, crossing the peritrophic matrix, which is a blocking layer around ingested food, which protects it against the mosquito's immune system during digestion, and many substances in the mosquito's midgut inhibit extrinsic immunity responses (Kajla *et al.*, 2015). The ookinete invasion stimulates the mosquito's midgut epithelium immunity response about 24 h after being fed infected blood to counter the *Plasmodium* invasion (Hoffman *et al.*, 2002; Vlachou *et al.*, 2006; Mueller *et al.*, 2009; Niamnuy, 2012). The immunity response is activated through a variety of routes, such as, the *STAT* pathway, to produce nitric oxide in order to eliminate any *Plasmodium* parasites that have infected cells in the midgut epithelium (Fig. 7). However, the high level of nitric oxide also affects the midgut epithelium by activating apoptosis and the *SOCS* gene is also induced to commence the transcription of mRNA to produce *SOCS* proteins which causes a negative feedback by inhibiting the *STAT* pathway (Han *et al.*, 2000).

These results indicate that the *SOCS* gene plays an important role in mosquito immunity. It causes a form of negative feedback to counter hyper-immunity
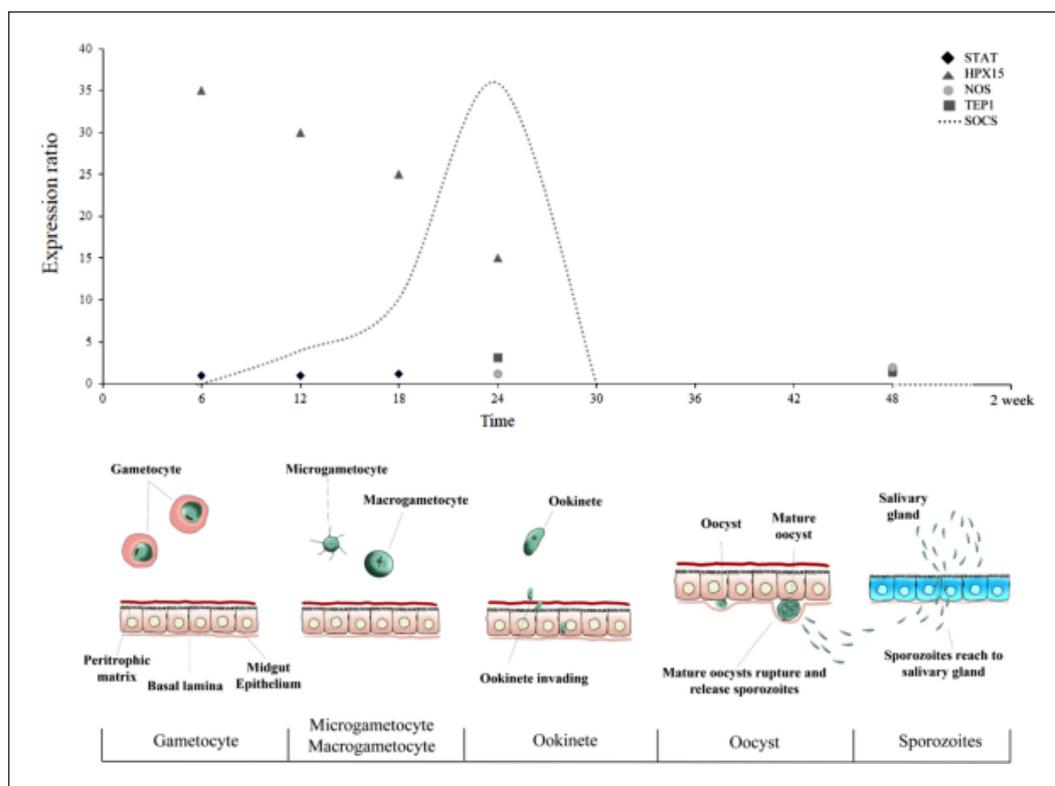
Figure 7. The sequence of the malaria parasite development stages in the mosquito and the mosquito's immune-system gene response supported by qPCR.

against *Plasmodium* invasion within 24 h of ingesting *Plasmodium* parasites. In this study, this caused the higher expression of the *SOCS* gene 24 h after feeding in the parasite-positive blood-feeding pool than in the negative blood and non-feeding pools. Moreover, *SOCS* gene expression was found at a higher level in the abdomen than in the thorax because the midgut epithelium in the abdomen represents the first barrier to counteract *Plasmodium* parasites. So, the *STAT* pathway was highly active in the midgut epithelium producing nitric oxide in order to eliminate the parasites but this in turn caused negative feedback which restrained the production of this toxic molecule (Waterhouse *et al.*, 2007; Bahia *et al.*, 2011). In the case of the expression of the *SOCS* gene after the mosquitoes were fed parasite-negative blood, although the blood was not infected with *Plasmodium* parasites, it was still able to stimulate *SOCS* gene expression as well as the expression of the *HPX15* gene, which is stimulated by either blood or sugar feeding. In a previous study of the expression of the *SOCS* gene in *An. culicifacies* infected with *P. berghei*. Dhawan *et al.* (2015) found that the *SOCS* gene was more highly expressed in the midgut 24 h after with *P. berghei*-infected blood than in other organs and the results of the present study are in agreement with Dhawan *et al.* (2015)'s finding.

However, the rapid expression of the *SOCS* gene may prevent the complete elimination of *Plasmodium* parasites. If *SOCS* gene expression decelerates or gene knockdown is used as a means of preventing its expression, this can reduce *Plasmodium* parasite invasion and inhibit the potency of the malaria vector. The *SOCS* gene has previously been suspected of supporting *Plasmodium* infection in *Anopheles* mosquitoes as has the *HPX15* gene (Kajla *et al.*, 2017) and this study's findings appear to support that role of the *SOCS* gene. However, more research into the interaction between mosquitoes and malaria parasites is needed since the identification of anti-malaria immune genes will lead to the potential control and prevention of malaria, not only in Thailand, but throughout the world.

CONCLUSION

This research supports the hypothesis that *SOCS* gene expression in *An. dirus* is related to *P. vivax* infection, because its stimulation plays a role in the mosquito's immune response against infection. Further research relating to gene expression associated with the immunity system of mosquitoes, particularly in relation to the response to *P. falciparum* and *P. ovale*, in *An. minimus*, and *An. Maculatus*, which are the other primary malaria vectors in Thailand, may uncover new means of controlling and preventing malaria in the future.

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