PROTEOMIC ANALYSIS OF ASEXUAL STAGES, YOUNG AND MATURE GAMETOCYTES OF *PLASMODIUM FALCIPARUM* STRAIN NF54 BY MASS SPECTROMETRY

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Abstract. *Plasmodium falciparum* has a complex life cycle within the human host and specialized proteins at each stage are required. A proteomic analysis and protein classification of asexual stages, young and mature gametocytes were conducted as a preliminary study towards a better understanding of the biology of this complex vector-borne parasite. *P. falciparum* strain NF54 was cultivated and asexual parasites, young gametocytes (Stages I-III) and mature gametocytes (Stages IV-V) were harvested on days 3, 8 and 13, respectively. Proteins expressed in each type of parasite preparation were separated by SDS-PAGE and identified by mass spectrometry. A total of 7,778 proteins were detected, of which 3,220 were exclusively in asexual stages, 463 from young gametocytes and 1,699 from mature gametocytes while 691 proteins were common to all three types of parasites. In order to obtain the functional characteristics of these parasite proteins, they were classified using a protein analysis through evolutionary relationships classification system (PANTHER). Receptor proteins were absent in young gametocyte while asexual stages had a five-fold higher content than mature gametocytes. Proteins with antioxidant activity in young gametocytes were five-fold more abundant than in both asexual parasites and mature gametocytes. Mature gametocyte showed a two-fold higher percent membrane-associated proteins than the other two parasite types. The data obtain from this study should be of use in more detailed investigations of proteins expressed in the various parasite stages, which could lead to discoveries of novel antimalarials and development of more effective vaccines.

Keywords: *Plasmodium falciparum*, asexual stage, gametocyte, mass spectrometry, proteome

INTRODUCTION

Malaria is one of the most serious public health problems worldwide due to its high mortality and morbidity in large parts of sub-Saharan Africa, tropical and subtropical regions of Asia and South America. The most severe form of malaria is caused by *Plasmodium falciparum* (Snow *et al*, 2005). According to the latest WHO estimates, 212 million cases of malaria occurred globally and an estimated 429,000 people died from malaria infection (WHO, 2016).

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P. falciparum has a complex life cycle; the parasite develops into a series of separate morphology in vertebrate and invertebrate hosts. In human host, both asexual and sexual stages of *P. falciparum* are found in erythrocytes (Sinden, 1985; Bruce *et al*, 1990). Sexual reproduction is an essential process in the parasite life cycle and occurs subsequent to transmission from the human host to the mosquito vector. Development of asexual to gametocyte stages takes about 12 days, involving five morphology stages, known as stage I to V. Stage I, a round form, does not fill the erythrocyte and a vacuole is not found in the cytoplasm; Stage II is triangular or irregular shape and slightly elongated; Stage III is elongated with slightly rounded ends; Stage IV is symmetrical, elongated, and spindle-shape with pointed ends, distorting the erythrocyte; and Stage V is sausage-shape with rounded ends and that containing scattered pigment is male and that containing dense pigment, pink to light violet is female (Sinden, 2009). Stage V gametocyte appears only in peripheral blood and thereby transmitted to mosquitoes, whereas the four earlier stages are found in the bone marrow and spleen (Hawking *et al*, 1971).

In 2002, the complete genome sequence of *P. falciparum* (clone 3D7) was determined (Gardner *et al*, 2002) providing an opportunity to study the transcriptome and proteome of the four major stages of the parasite life cycle, namely, sporozoite, merozoite, trophozoite and gametocyte. In *P. falciparum* 3D7 over 2,400 proteins have been identified, with 1,427 in early gametocytes (Stage I-II) and 2,031 in mature gametocyte (Stage V) (Silvestrini *et al*, 2010). In another known *in vitro* gametocyte-producing strain, NF54, proteomic analysis of different developing stages of the parasites revealed 1,289 proteins, of which 714 proteins are expressed in asexual stages, 931 in gametocyte, and 645 in gametes and it is the only proteomic study to date of the different stages (Lasonder *et al*, 2002). However, the number of gametocyte proteins is much lower than previous report for *P. falciparum* 3D7. Although the proteomes of other *Plasmodium* gametocytes have been reported (Florens *et al*, 2002; Hall *et al*, 2005; Khan *et al*, 2005; Silvestrini *et al*, 2010), however, the protein classification of young and mature gametocytes has not been investigated.

Hence, in this study, young (Stage I-III) and mature *P. falciparum* gametocytes (Stage IV-V) from *in vitro* culture were subjected to proteomic analysis using mass spectrometry in comparison to the asexual stages for a better understanding of the biochemical processes among parasite stages.

MATERIALS AND METHODS

Parasite cultivation

P. falciparum strain NF54 was obtained from Mahidol Vivax Research Center, Faculty of Tropical Medicine, Mahidol University, Bangkok and grown in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% human serum and human red blood cell (group O, Rh+) at 37ºC under an atmosphere of 5% CO_2 (Chavalitshewinkoon and Wilairat, 1991). When 10% parasitemia was reached, the culture was synchronized at the ring stage using 5% D-sorbitol (Lambros and Vanderberg, 1979) and parasites were cultured at an initial 1% parasitemia. Culture medium was changed daily by removal of used and addition of fresh medium pre-warmed at 37ºC and parasites were grown continuously using treated

erythrocytes (Chavalitshewinkoon and Wilairat, 1991). Parasite morphology and percent parasitemia were observed under a light microscope.

Cultivation of young and mature gametocytes

Gametocyte cultivation was initiated with 1% parasitemia at ring form in 2% erythrocyte suspension in a culture flask. Medium was changed on day 4. In order to obtain young gametocytes, culture was centrifuged at 500*g* for 7 minutes, then 5 volumes of 5% D-sorbitol solution were added and culture was further incubated for 5 minutes at 37°C. Then, the culture was washed twice with medium and further incubated at 37°C for two days. On days 6 and 7 of cultivation, erythrocytes were sedimented at 500*g* for 7 minutes at room temperature and re-suspended in RPMI 1640 medium supplemented with 50 mg/l hypoxanthine, then the culture was flushed for 1 minute with a gas mixture of 5% CO_2, 5% O_2, and 90% N_2. The young gametocytes (Stages I-III) were harvested on day 8. Preparation of mature gametocytes (Stages IV-V) was conducted using the same procedure for young stages except 5% D-sorbitol treatment was performed on three consecutive days (days 6-8) and the mature stages were harvested on day 13 (Petmitr *et al*, 1995). Infected red blood cells of each preparation were centrifuged at 500*g* for 10 minutes at 4°C and parasites were released from infected erythrocytes by adding an equal volume of 0.05% saponin in phosphate-buffered saline (PBS) to pellet and incubating at 37°C for 30 minutes. Parasite pellet was washed twice with ice-cold PBS, or until supernatant was clear. Parasites were kept at -80°C for further analysis.

Sample preparation for proteomic analysis

Erythrocytes containing asexual parasites, and isolated young and mature gametocytes were adjusted to ~$7x10^7$cells in 0.5% SDS solution containing a proteinase inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA). Parasite solution was kept on ice for 30 minutes and then mixed by vortexing. The protein solution was dialyzed against phosphate buffer saline (PBS) using a 3,500 kDa cut-off dialysis bag for 24 hours at 4°C and kept at -20°C until used. Protein concentration was measured using Bradford's method (Bradford, 1976) and adjusted to 150 mg/μl. Protein solution was mixed with 2X loading solution [100 mM Tris-Cl (pH 6.8) 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol and 200 mM dithiothreitol (DTT)], heated at 90°C for 5 minutes, and centrifuged the samples at 8,000*g* at 4°C for 5 minutes. Supernatant proteins were separated by 12% SDS-PAGE and stained with Coomassie brilliant blue dye.

A protein band was excised from the gel, cut into small pieces, then added to 500 μl of 50 mM iodoacetamide and 500 μl of acetonitrile and incubated at room temperature for 30 minutes. Liquid was replaced with 100 μl of 4 mM DTT in 50 mM ammonium bicarbonate and incubated at 60°C for 15 minutes. The gel pieces were cooled to room temperature and 7 μl aliquot of 250 mM iodoacetamide in water was added and kept in the dark for 30 minutes. Then 3 μl aliquot of the above DTT solution was added, mixed and the solution discarded. The gel pieces were dehydrated by adding 200 μl of acetonitrile, incubating at room temperature for 15 minutes and drying for 10 minutes before being digested with 2 μM trypsin at room temperature for 15 minutes. Then 50 μl of 5% acetonitrile and 200 μl of 50 mM ammonium bicarbonate were added and the gel suspension incubated at 37°C

overnight, added with 300 µl of acetonitrile and incubated at room temperature for 30 minutes. The supernatants were kept at -20°C for further analysis.

Mass spectrometry (MS) analysis

The in-gel trypsin-digested proteins were dried using a speed vacuum system (Tomy, Tokyo, Japan), then 0.1% formic acid was added and the sample introduced into an UltiMate 3000 nano-LC system (Dionex, Surrey, UK) coupled with a micrOTOF-Q (Bruker Daltonics, Bremen, Germany). Data acquisitions were obtained using a Hystar software (Bruker Daltonics, Bremen, Germany). The LC-MS/MS data were analyzed using a Mascot version 2.4.1 (Matrix Science, London, UK) and NCBInr database (www.ncbi.nlm.nih.gov). *P. falciparum* was set as the taxonomy filter, with all reported proteins having at least 95% confidence level. PANTHER software was used to classify the proteins identified according to gene ontology (Mi *et al*, 2013).

RESULTS

Parasite preparations

Approximately 5% parasitemia was obtained at day 3 of parasite cultivation containing 1.7% ring forms, 2.0% trophozoites and 0.3% schizonts (Fig 1A); there were no gametocytes. On day 8, after

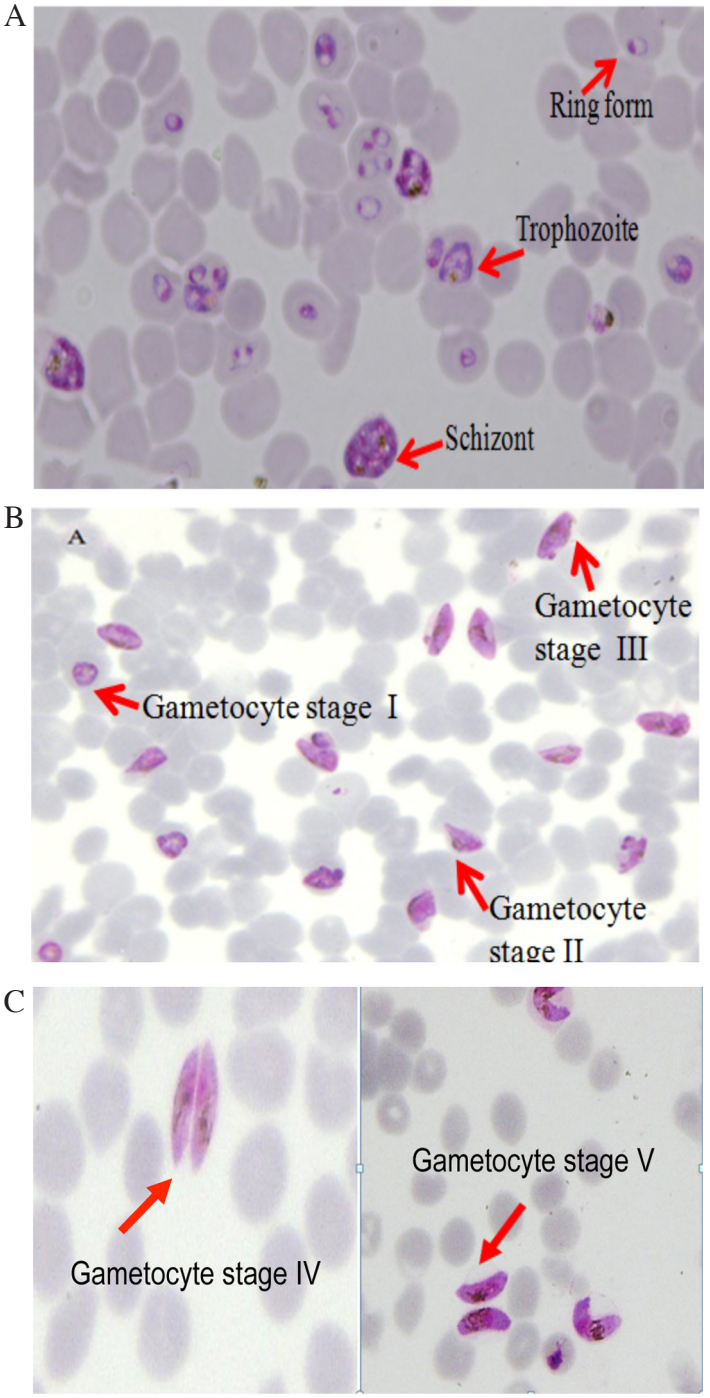

Fig 1–Morphology of asexual stages and gametocytes of *Plasmodium falciparum* strain NF54. Giemsa-stained slides of asexual parasites on day 3 of cultivation (A), young gametocytes on day 8 (Stages I-III) (B) and mature gametocytes (Stages IV and V) on day 13 (C) are shown.

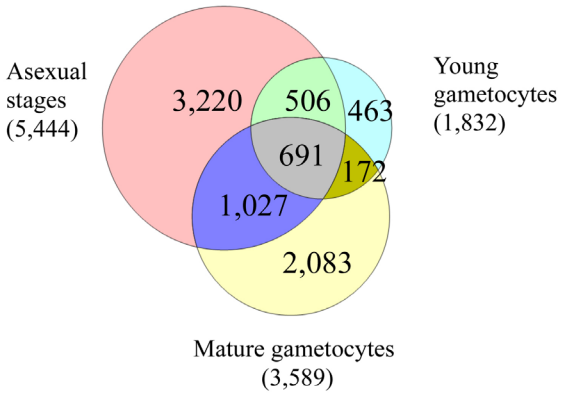

Fig 2–Venn diagram of numbers of *Plasmodium falciparum* strain NF54 proteins of asexual stages and young and old gametocytes. The number of proteins in each parasite category is shown in brackets.

5% D-sorbitol treatment for three consecutive days, pure young gametocytes (Stages I-III) at 6.5% parasitemia were obtained and the number of gametocytes stage III was two folds higher than the other two stages (Fig 1B). The elongated form of Stage II, is only observed in *P. falciparum*, while gametocytes of most other mammalian *Plasmodium* species are round cells (Silvestrini *et al*, 2010). Mature gametocytes (Stages IV and V) at 5.1% parasitemia were harvested on day 13 (Fig 1C). Stage IV gametocyte was identified as symmetrical, elongated, and spindle-shape with pointed ends, while Stage V gametocyte (most mature form) showing a sausage shape with pigments. Approximately 0.2 ml of parasite pellet could be obtained from four culture flasks.

Parasite proteomes

A total of 7,778 proteins from the asexual and sexual stages of *P. falciparum* NF54, 5,444 proteins from the asexual stages, 1,832 from young gametocytes and 3,589 from mature gametocytes (Fig 2). Some 691 proteins were shared among all three types of parasite preparations. The asexual stages contained exclusively 3,220 proteins, whereas 2,718 proteins were found only in gametocytes (young and mature), 863 of which were present in both young and mature gametocytes, and 2,224 proteins were present in both gametocytes and asexual stages.

Using PANTHER-based ontology, 5,444, 1,832 and 3,589 proteins of asexual stages, young gametocytes and mature gametocytes, respectively were analyzed for their molecular function, biological process and cellular compartment. According to their molecular functions, the majority (76-81%) of proteins expressed in all three parasite types were enzymes and receptors (Fig 3). Interestingly, the percent proteins with receptor function in asexual parasites was five folds higher than mature gametocyte and these types of proteins were not detectable in young gametocytes. Proteins related to antioxidant activity in young gametocytes were five-fold more abundant than other two types of parasite preparations. Only 2% of proteins in young gametocytes were responsible for transporter function compared with 6% and 5% found in asexual stages and mature gametocytes, respectively.

Nine biological processes were identified in all three parasite preparations, with 79-81% involved in metabolic, cellular and biological regulation functions (Fig 4). However, there was no difference in percent proteins in the four cellular components found among three types of parasite stages except for a two-fold higher percent proteins in membranes in mature gametocytes (Fig 5).

Proteins of the asexual stages, young and mature gametocytes could be grouped into 23, 20 and 22 classes, respectively

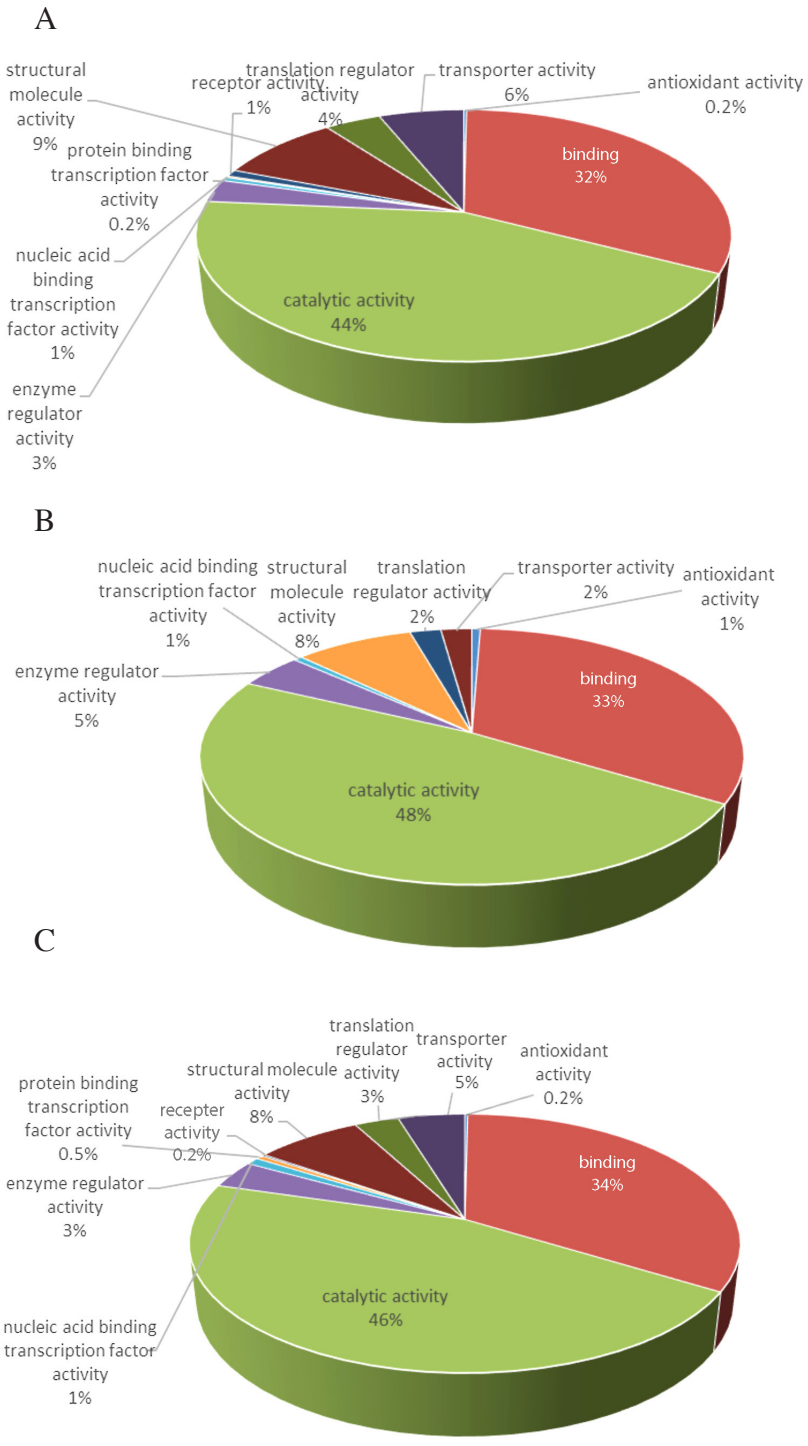

Fig 3– Pie charts of *Plasmodium falciparum* strain NF54 protein distribution according to molecular functions of asexual stages (A), young gametocytes (B) and mature gametocytes (C).

(Fig 6). Four protein classes were not found in young gametocytes, namely receptor, structural proteins, defense/immunity proteins and isomerases. Interestingly, transmembrane receptor regulatory/adaptor proteins were observed in young and mature gametocytes but not found in the asexual stages. The top five protein classes of asexual stages were nucleic acid binding (30.4%), hydrolase (12.9%), transferase (9.5%), cytoskeletal protein (7.8%), and enzyme modulator (6.0%), respectively. However, kinase was the fourth protein class in rank of young (8.8%) and mature (6.5%) gametocytes.

DISCUSSION

In this study, the proteomes of *P. falciparum* NF54 asexual stages, young and mature gametocytes were identified by LC-MS/MS in combination with NCBInr database. The total number of parasite proteins identified and classified were 4.7 folds higher than proteins (714 in asexual stage and 931 in gametocytes) previously reported from the same parasite strain (Lasonder

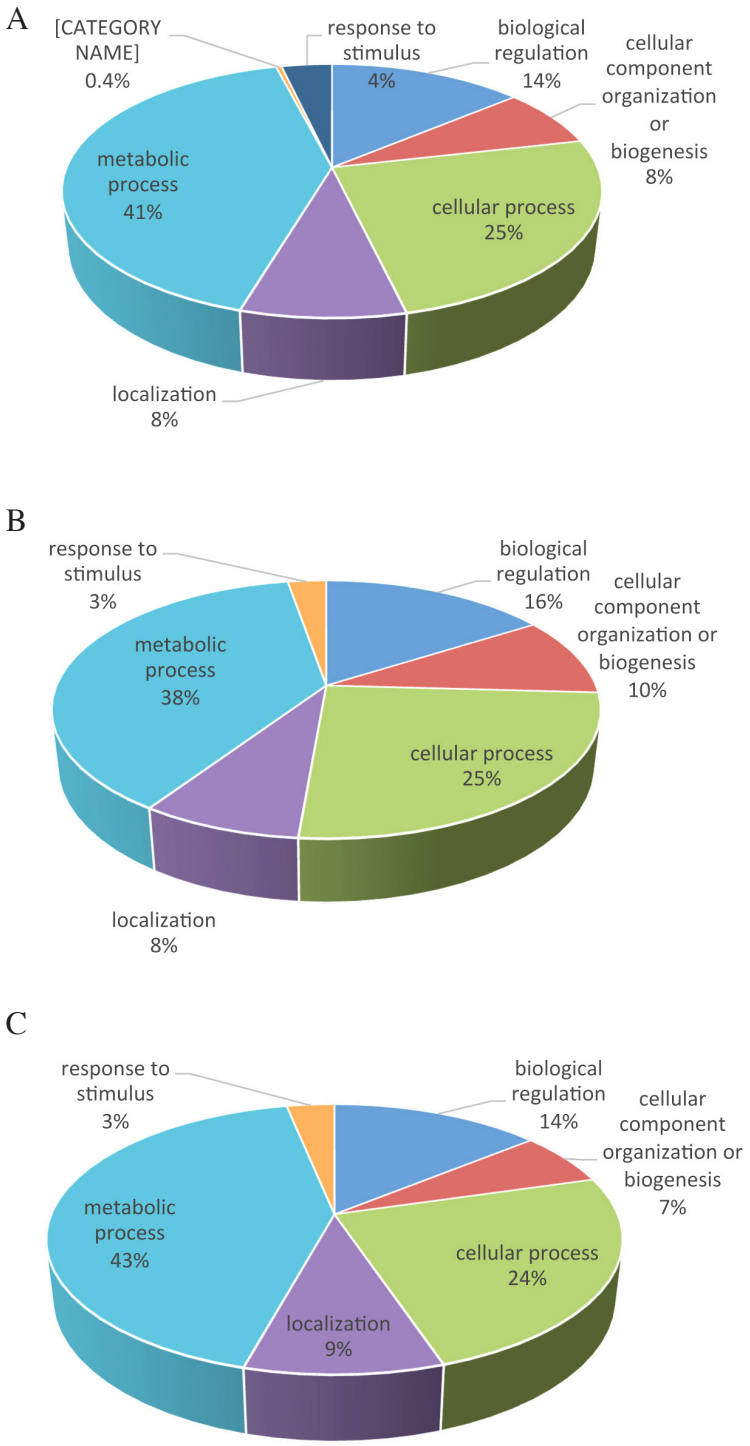

Fig 4–Pie charts of *Plasmodium falciparum* strain NF54 protein distribution according to biological processes found in asexual stages (A), young gametocytes (B) and mature gametocytes (C).

et al, 2002) and 1.6 folds higher than those (1,345 in trophozoite, 1,427 in young gametocyte and 2,031 in old gametocyte) of *P. falciparum* 3D7 (Silvestrini *et al*, 2010). The larger number of parasite proteins identified in our study could be attributed to the greater amount of parasite proteins used in the analysis (approximately 6 folds higher than in the previous study).

The much lower number of receptor proteins found in young gametocytes is consistent with previous experiments showing a much reduced production of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) in early gametocytes compared to asexual trophozoites, with a minimal exposure of such adhesins on the erythrocyte surface (Tibúrcio *et al*, 2013). But which kinds of surface proteins/glycoproteins young gametocytes employ to sequester in the bone marrow (or elsewhere) remain unclear.

The higher abundance of antioxidant proteins found in gametocytes compared with the asexual stages is related to the up-regulation of the pro-

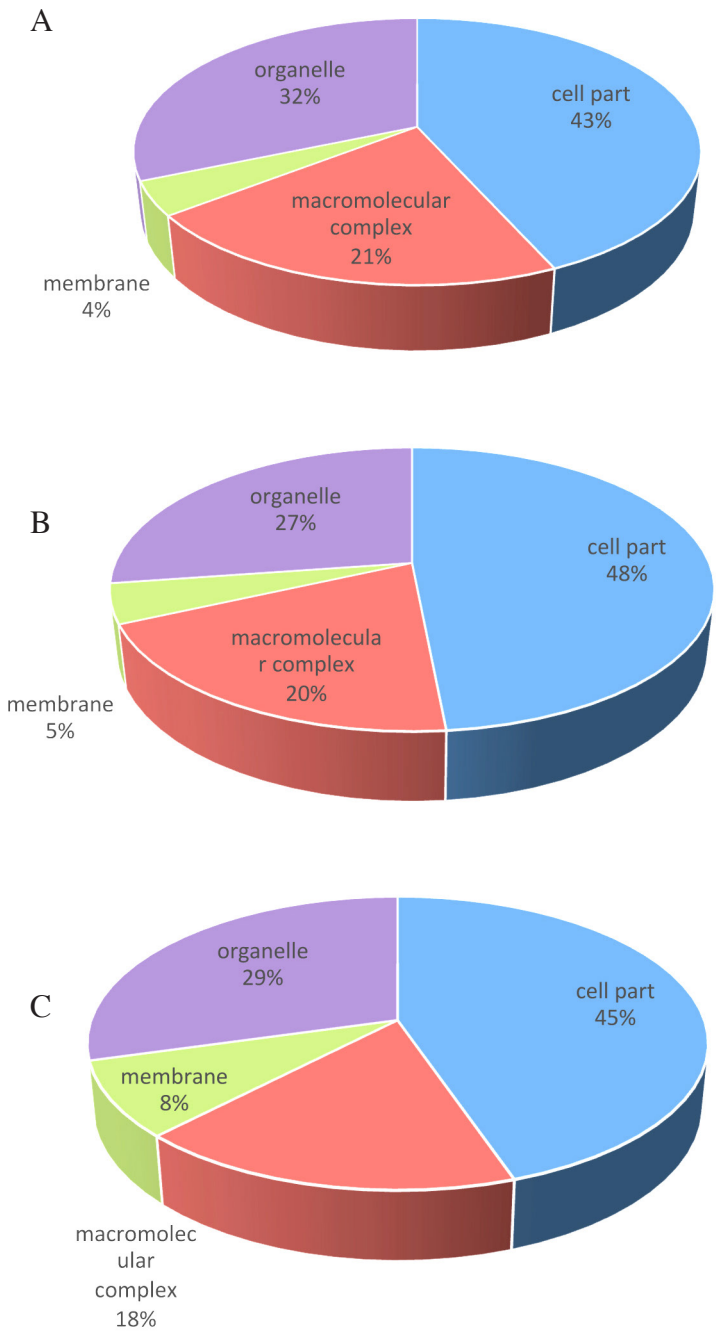

Fig 5–Pie charts of *Plasmodium falciparum* strain NF54 proteins distribution according to cellular components of asexual stages (A), young gametocytes (B) and mature gametocytes (C).

teins involved in protection against oxidant stress, such as glyoxalases I and II, and two different glutaredoxins in gametocytes and gametes (Rahlfs *et al*, 2001). Moreover, enzymes involved in the synthesis and metabolism of glutathione, for instance glutathione *S*-transferase and γ-glutamylcysteine synthetase, are exclusively present in the sexual stages (Lasonder *et al*, 2002).

Sexual stage-specific proteins located on gametocyte parasitophorous vacuole (PV) are abundantly produced during 40-48 hours of gametocytes maturation (Eksi *et al*, 2005; Alano, 2007), contributing to the higher abundance of transmembrane proteins found in young and mature gametocytes. In addition, protein Pfpeg3 is highly present in all subcellular compartments derived from the gametocyte PV membrane (Furuya *et al*, 2005; Silvestrini *et al*, 2005, Lanfrancotti *et al*, 2007). Disruption of *Pfpeg3* results in a significant reduction in gametocyte production, and disruption of the internal membrane organization of developing gametocytes, particularly in males (Furuya *et al*, 2005). In addition, gametocyte PV might have specific features related to a distinctive trafficking machinery active in the sexual parasite (Alano, 2007) and

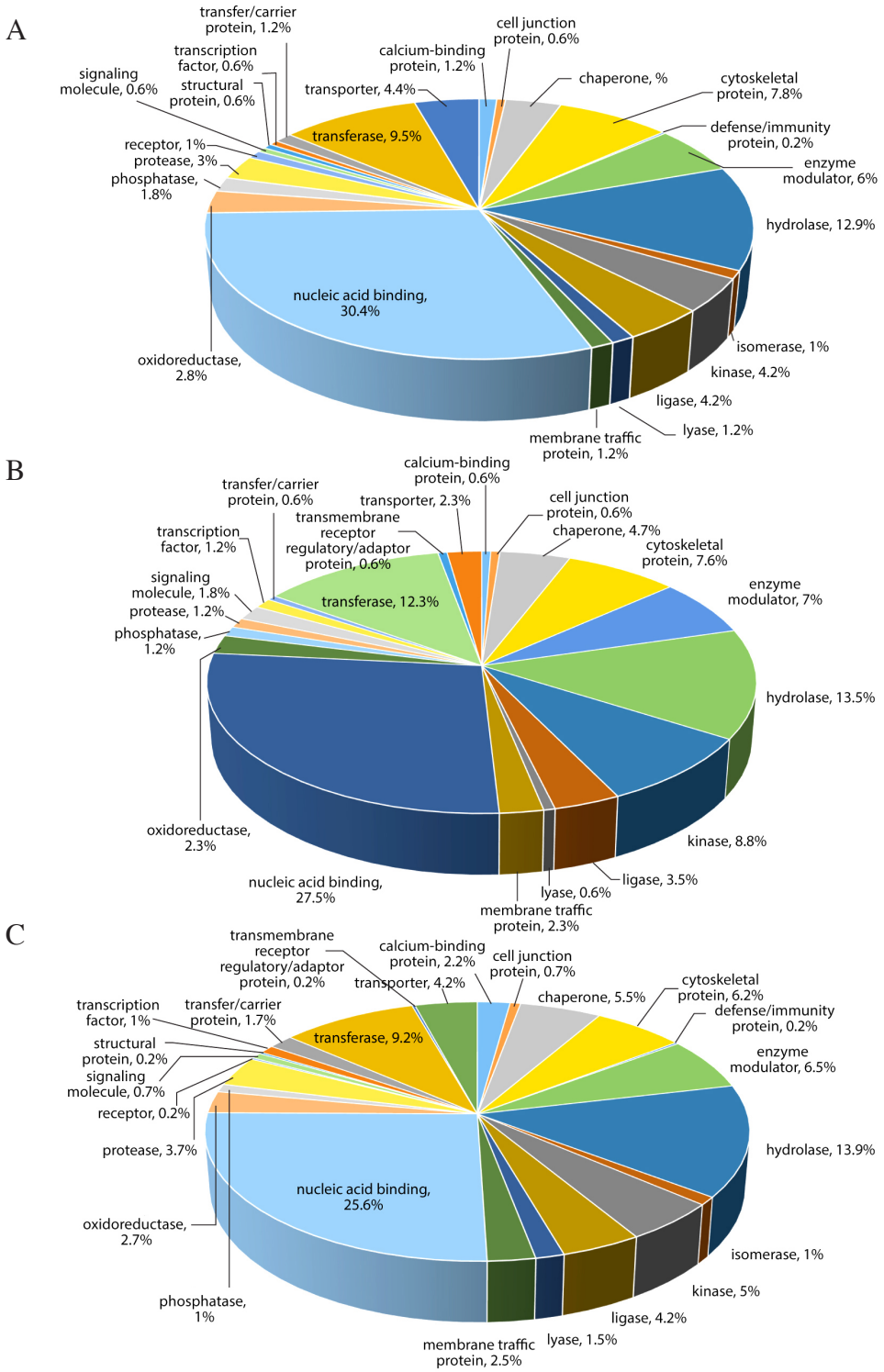

Fig 6–Pie charts of *Plasmodium falciparum* strain NF54 protein distribution according to their classifications based on PANTHER (http://www.pantherdb.org) of asexual stages (A), young gametocytes (B) and mature gametocytes (C).

the process might have specific differences from the egress of asexual schizonts (Salmon *et al*, 2001; Wickham *et al*, 2003).

Higher number of protein kinases obtained in both young and mature gametocytes is consistent with a previous report that five additional protein kinases are identified and upregulated in female gametocytes and 10 additional kinases are upregulated in male gametocytes (Tao *et al*, 2014).

The detailed proteome profiles described in this communication provide a springboard from which to launch more detailed comparative studies of the biochemical processes between asexual and sexual forms of *P. falciparum*, which should be useful in the development of novel antimalarials and more effective vaccines.

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