METHOD ARTICLE

**An enhanced toolkit for the generation of knockout and marker-free fluorescent *Plasmodium chabaudi* [version 2; peer review: 2 approved]**

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Abstract

The rodent parasite *Plasmodium chabaudi* is an important in vivo model of malaria. The ability to produce chronic infections makes it particularly useful for investigating the development of anti-*Plasmodium* immunity, as well as features associated with parasite virulence during both the acute and chronic phases of infection. *P. chabaudi* also undergoes asexual maturation (schizogony) and erythrocyte invasion in culture, so offers an experimentally-amaenable in vivo to in vitro model for studying gene function and drug activity during parasite replication. To extend the usefulness of this model, we have further optimised transfection protocols and plasmids for *P. chabaudi* and generated stable, fluorescent lines that are free from drug-selectable marker genes. These mother-lines show the same infection dynamics as wild-type parasites throughout the lifecycle in mice and mosquitoes; furthermore, their virulence can be increased by serial blood passage and reset by mosquito transmission. We have also adapted the large-insert, linear PlasmoGEM vectors that have revolutionised the scale of experimental genetics in another rodent malaria parasite and used these to generate barcoded *P. chabaudi* gene-deletion and –tagging vectors for transfection in our fluorescent *P. chabaudi* mother-lines. This produces a tool-kit of *P. chabaudi* lines, vectors and transfection approaches that will be of broad utility to the research community.

Keywords

Plasmodium chabaudi, malaria, Transfection, PlasmoGem
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Introduction

The rodent parasite *Plasmodium chabaudi* is a leading in vivo model for the study of host immunity and immunopathology in malaria, and provides an extremely powerful tool to study the parasite biology and parasite-host interactions that underpin virulence, chronicity and transmission (Spence et al., 2013; Spence et al., 2015). This is because *P. chabaudi* demonstrates sequestration and rosetting at the mature schizont stage and resolves to establish a chronic infection with periodic recrudescence and antigenic variation, in a similar manner to the dominant cause of human malaria mortality, *P. falciparum* (reviewed in (Langhorne et al., 2002; Stevenson & Riley, 2004)). Furthermore, commitment to sexual development (gametocytogenesis) increases after the resolution of acute infection, and development in the mosquito progresses through each bottleneck with physiologically relevant parasite numbers (Spence et al., 2012). In addition, there are a large number of phenotypically diverse parasite genotypes available, which provide a unique opportunity to a) develop co-infection, superinfection and re-infection models and b) interrogate the role of parasite genetics in shaping the course and outcome of infection. *Plasmodium chabaudi* is therefore an extremely adaptable model that can be used to study every step in the *Plasmodium* life cycle.

Despite the phenotypic advantages of this model system, the development of transfection technologies for *P. chabaudi* has lagged behind those for other rodent malaria models. Methods for generating *Plasmodium chabaudi* reporter lines by single cross-over recombination and genomic integration of a fluorescent or luminescent protein cassette have, however, been described (Brugat et al., 2014; Reece & Thompson 2008; Spence et al., 2011), and these parasites and transfection methods have proved useful for imaging parasite accumulation and associated pathology in host tissues (Brugat et al., 2014; Borges da Silva et al., 2015; Brugat et al., 2017; Cunningham et al., 2017; Medeiros et al., 2013), as well as for visualising parasite protein localisation (Yam et al., 2016). Nevertheless, the relative inefficiency of these techniques has hindered the progress of *P. chabaudi* experimental genetic studies.

To improve the accessibility and reproducibility of *P. chabaudi* transfection techniques, we have optimised methods that enable parasite purification and transfection in the laboratory and drug selection in wild-type mice, and have used these methods to generate stable, highly-fluorescent lines by double cross-over integration of genes encoding green- and mCherry-fluorescent proteins (Shaner et al., 2004) into a dispensable gene locus, using a transfection vector that allows recycling of the drug-selectable marker. These parasites are viable throughout the lifecycle in mice and mosquitoes and can be used as a fluorescence mother-line. We tested this by adapting high efficiency large-insert *PlasmoGEM* vectors and used them to generate gene-modified fluorescent parasite lines (Pfander et al., 2011). Together, this toolkit provides a clear way forward for future expansion of gene targeting studies in *P. chabaudi*.

Methods

Ethical statement and mouse procedures at the University of Edinburgh

All procedures were carried out in accordance with the UK Home Office regulations (Animals Scientific Procedures Act, 1986; project licence number 70/8546 and P04ABDCAA) and approved by Ethical Review Body of the University of Edinburgh. Throughout this study, all efforts were made to reduce animal usage and ameliorate harm to animals. Mice were housed in the University of Edinburgh Licensed Animal Facilities 60/2605, and all animal procedures were performed in laboratories within the animal facilities. C57Bl/6Jax mice were bred under specific pathogen free conditions at the University of Edinburgh. Experimental female C57Bl/6 mice, aged six to twelve weeks, were kept in specific-pathogen-free conditions and subjected to regular pathogen monitoring by sentinel screening. Mice were housed with at least one companion in individually ventilated cages furnished with autoclaved woodchip, fun tunnel and tissue paper at 21 ± 2°C under a reverse light-dark cycle (light, 19.00 – 07.00; dark, 07.00 – 19.00) at a relative humidity of 55 ± 10%. Mice were housed under these light-dark cycle conditions to allow collection of *P. chabaudi* trophozoites prior to schizogony at 13.00-15.00 hrs, and were allowed to adapt to a reverse-light schedule for at least 7 days before *P. chabaudi* infection. They were fed a commercially available, autoclaved dry rodent diet (Rat and Mouse No. 3 Breeding diet; Special Diets Services) and water, both available ad libitum. The health of mice was monitored by routine daily visual health checks, and *P. chabaudi*-infected mice were monitored at least twice daily.

Mice, mosquitoes and parasites

The C57Bl/6 mouse *Plasmodium chabaudi chabaudi* AS animal model of malaria was chosen to minimize host genetic variability and to obtain robust infections with a very low incidence of severe disease. *P.c. chabaudi* AS parasites were obtained from the European Malaria Reagent Repository at the University of Edinburgh. *Anopheles stephensi* were maintained, and transmission of *P. chabaudi* was carried out, as described in (Spence et al., 2012; Spence et al., 2013). All experimental groups consisted of 2-6 mice housed together. Transfection experiments required 10 mice; two mice to provide infected blood for transfection and eight mice as recipients of parasites transfected with four plasmids in replicate. Parasite cloning required six mice and phenotypic experiments were carried out using groups of 7-8 mice, to provide statistical significance. Euthanasia was performed by cervical dislocation at the end of phenotypic experiments, or by exsanguination under anaesthesia (pentobarbital sodium; Euthatal). Mice were exsanguinated to obtain...
infected and uninfected blood for transfection experiments, or to collect genetically-modified parasites for storage as stables in liquid N. This specific method of anaesthesia reduces animal suffering whilst maximising blood volume obtained.

Monitoring of infection
Female C57Bl/6jax mice, aged 6–10 weeks with median weight of 22.5g (IQR 21.7g-23.1g), were inoculated ip with 1×10⁷ PcAS-GFP-infected red blood cells and monitored for parasitaemia, weight loss and anaemia daily. Parasitaemia was enumerated either on giemsa-stained, thin-blood smears or by flow cytometric analysis (described in Underlying data; Thompson, 2020). In the latter case, 2μL of blood was diluted in 1mL of KSG-H (0.1 M NaCl, 4.6 mM KCl, 1.2 mM MgSO4·7H2O, 25 mM NaH2PO4, 0.2% (wt/vol) glucose, 25 IU ml⁻¹ heparin sodium), made as described in (Spence et al., 2011), maintained at 4°C and then diluted a further 1:5 prior to acquisition (150,000 events per sample) on a BD Fortessa analyser (Becton Dickinson, UK) within 2 hours of sampling. Using FlowJo analysis software (Becton Dickinson, UK), gating was performed on singlets (FSC-A v FSC-W), cells (FSC-A v SSC-A) and native GFP (B530/30nm). Free Flow software, FCalyzer, is available at https://sourceforge.net/projects/fcsalyzer/. GFP gates were set using uninfected blood, with a median of 0.06% background signal and a limit of detection threshold of 0.85% (n=42). For anaemia, haemoglobin (g/L) was determined by Hemocue Hb201+ analyser (Radiometer, Sweden) with moderate anaemia defined as 80-120 g/L and severe anaemia as <80 g/L, in accordance with WHO guidelines. The mean haemoglobin concentration in 36 uninfected control mice was 140 g/L (data was collected from mice at the point when they were assigned to experimental groups in this study).

Generation of circular transfection vectors
pCAT-230p-G6 was generated by replacing the sil6 target regions of pBAT-SIL6-G6 (Kooij et al., 2012) with bps 640-1680 and 3312-4255 of the P . c. chabaudi 230p (PCHAS_0308200) locus. The 230p target regions were amplified from P . c. chabaudi AS genomic DNA using primers 5’230pF x 5’230pR (5’ target region) and 3’230pF x 3’230pR (3’ target region) and cloned into the multiple cloning sites of pBAT-Sil6-G6 or –M6 to generate pCAT-230p-G6 or pCAT-230p–M6. Plasmids to delete genes encoded by the PcG01 and PcG02 library clones were subsequently modified by site-specific recombineering mediated by 50 bp homologous targeting regions so to delete or disrupt the open reading frame (knock-out vectors), or delete the stop codon (tagging-vectors). The recombineering step introduces a C-terminal tagging vectors from the mapped PcG01 and PcG02 library using a custom software pipeline as previously described for generating a P. berghei resource (Schwach et al., 2015).

Plasmids and protocols for the parallel recombinease-mediated engineering (recombineering) of PcG01 and PcG02 library clones on 96-well plates were the same as used previously for the generation of P. berghei vectors (Pfander et al., 2011). Briefly, (1) TSA cells harboring PcG01 and PcG02 library clones were made transiently recombineering-competent by introduction of the pSC101gbdA plasmid that encodes elements of the red/ET operon of bacteriophage lambda (Zhang et al., 1998), (2) the target genes encoded by the PcG01 and PcG02 library clones were subsequently modified by site-specific recombineering mediated by 50 bp homologous targeting regions so to delete or disrupt the open reading frame (knock-out vectors), or delete the stop codon (tagging-vectors). The recombineering step introduces a dual (zeo-pheS) bacterial selectable marker which confers resistance to zeocin and sensitivity to 4-chloro-DL-phenylalanine. (3) In a LR-Gateway Clonase I (Thermofisher, catalogue # 11791042) mediated reaction with the pR6K attL1-3xHA-hdHfr/nco-attL2 plasmid, which also introduces a 3xHA epitope tag (silent in KO parasites), the zeo-pheS selection cassette was then replaced by a mutated version of human dihydrofolate reductase (hdhfr) coupled to uridyl phosphoribosyl
transferase (yFCU), which confers resistance to pyrimethamine and sensitivity to 5-fluorocytosine (5-FC) in *Plasmodium*. The *hdhfr*-yFCU cassette in pR6K attL1-3xHA-hdhfr-yfcu-attL2 (Pfander et al., 2011) is flanked by duplicated *hdhfr* 3′ UTR sequences, facilitating efficient recombination under 5-FC pressure.

Final vectors were subjected to full-length Illumina sequencing on a MiSeq platform to confirm the correct sequence of the homology arms before uploading design data to our public resource at https://plasmogem.sanger.ac.uk (Schwach et al., 2015).

SC101gbdA was propagated at 30°C in DH10B cells in 2x low salt Luria Broth (LB) with 5 µg/ml tetracycline, or together with plfaz clone plasmids in TB medium with 0.4% glycerol + 30 µg/ml kanamycin + 5 µg/ml tetracycline in TSA cells. The SC101gbdA and pR6K attR1-zeo³-Phe³-attR2 plasmid served as template for the amplification of the zeo-PheS cassette for the recombineering step. These plasmids were kind gifts from Francis Stewart, Dresden. The Gateway donor plasmid pR6K attL1-3xHA-hdhfr-yfcu-attL2 was the same as used for *P. berghei* PlasmoGEM vectors (Pfander et al., 2011). The R6K origin of replication plasmids were propagated in PIR1 (Invitrogen) bacteria in LB Broth with 5 µg/ml tetracycline.

**Transfection**

For transfection, plasmid DNA was prepared using Qiagen plasmid midi-kit (catalogue # 12143). Circular vectors were linearised with restriction enzymes AatII and ApaLI (New England Biolabs) and purified away from the plasmid backbone using gel electrophoresis and a Qiagen QiAquick gel purification kit (catalogue # 28704), according to the manufacturer’s instructions. DNA was resuspended in dH₂O at 0.2-1 µg/µl. The *PlasmoGEM* transfection vectors used to delete *pccrpm1* (PCHAS_0812700), *pccrpm2* (PCHAS_0617600), *pccrpm3* (PCHAS_0608500), *pccrpm4* (PCHAS_1304000) and PCHAS_0418000 were PGEM-600068, PGEM-597067, PGEM-596604, PGEM-610764 and PGEM-594684, respectively. The *PlasmoGEM* vectors were propagated in *E. coli* TSA cells (Lucigen, USA) according to *PlasmoGEM* protocols (Pfander et al., 2011; Pfander et al., 2013) and inserts were released by NotI digestion, followed by standard ethanol precipitation.

**P. chabaudi schizont culture**

The culture of *P. chabaudi* trophozoites to mature schizonts was carried out exactly as described in (Spence et al., 2011) with the following modifications. To provide parasites for culture, a single wild-type donor C57Bl/6Jax mouse (female, 6–10 weeks) was injected iv into a single wild-type recipient C57Bl/6Jax mouse (female, 6–10 weeks) as described (Spence et al., 2011). From 24 hours after transfection, recipient mice were supplied with acidified drinking water supplemented with pyrimethamine (35 µg/ml), refreshed every day for 5–12 days until parasites were visible. Mice were exsanguinated by cardiac puncture under terminal anaesthesia to collect infected blood. Correct integration of the resulting *PcAS-GFP*A230p and *PcAS-mChΔA230p* parasites (and deletion of 230p) was confirmed by diagnostic PCR using Phusion Blood Direct PCR kit (ThermoFisher Scientific; catalogue # 15280114). All PCR reactions were carried out using an MJ Research DNA Engine, PTC-200. 50 µl reaction mixtures contained 25 µl 2x Phusion Blood PCR buffer, 1 µl (0.5 µM) Primers F and R, 1 µl Phusion Blood DNA polymerase, 1 µl whole blood, 22 µl dH₂O. The cycling protocol was: one cycle x 98°C, 5 min; 40 cycles x 98°C, 1 sec, 72°C (anneal and extend), 30sec; one cycle x 72°C, 1 min.

**Recycling of the drug selection cassettes**

Following confirmation of plasmid integration into the 230p locus, 1 x 10⁷ PcAS-GFP*A230p* parasites were injected iv into the homology arms before uploading design data to our public resource at https://plasmogem.sanger.ac.uk (Schwach et al., 2015).
four wild-type C57Bl/6Jax mice. After 24 h (one cycle of replication), mice were supplied with the pro-drug 5-fluorocytosine in the drinking water (0.5 mg/ml) for 5 days until the parasitaemia of the recycled parasites reached 0.15%. Mice were exsanguinated by cardiac puncture under terminal anaesthesia to collect infected blood.

Recombination-mediated removal of the drug selectable cassette was determined by diagnostic PCR as described above (Phusson Blood Direct PCR Kit) and the ratio of recombined (primers recF x recR amplicons) to non-recombined parasites (dF x dR amplicons) was compared after 20, 25 and 30 cycles of PCR (Figure 1D). PcAS-GFP<(_{ML}> parasites showing a ratio > 2:1 recombined:non-recombined parasites were cloned by limiting dilution. Complete recombination and removal of hDHFR was then confirmed by diagnostic PCR as described above using primers recF x recR and dFxdr. A full list of primers used are provided in Table 1.

**Results**

**Design of transfection vectors**

P230p has been reported to be dispensable for asexual parasite replication in *P. falciparum*, *P. berghei*, *P. yoelii* and *P. knowlesi* (Hart *et al.*, 2014; Moon *et al.*, 2013; Thomas *et al.*, 2016; van Dijk *et al.*, 2010). To generate parasites that can be used as fluorescent reference lines throughout the *P. chabaudi* lifecycle, we modified the pBAT-SIL6 plasmid set (Kooij *et al.*, 2012) that contain cassettes encoding a pyrimethamine-selectable marker (hDHFR) to enable positive selection for transfectants, as well as positive selectable markers yeast cytosine deaminase and uridyl phosphoribosyl transferase (yFCU; confers sensitivity to 5-fluorocytosine (5-FC)) flanked by *pbdhfr-ts* 3’utr duplicate sequences, which promote recombination and recycling of both drug-selectable markers after negative selection with 5-FC (Figure 1B). Plasmids pCAT<(_{250p}>-G6 and pCAT<(_{250p}>-M6 are designed to recombine within the *P. chabaudi* 230p locus and introduce genes encoding green or mCherry fluorescent proteins, expressed under the control of the strong and constitutive *P. berghei* HSP70 5’ UTR promoter.

**Generation of stable, marker-free fluorescent *P. chabaudi* mother-lines**

Transfection in rodent malaria parasites relies on electroporation of merozoites (the extracellular stage of the blood cycle), which become mechanically released from arrested schizonts during the isolation procedure, and then subsequently re-invasive host reticulocytes or erythrocytes after injection into the bloodstream of a recipient mouse. It is likely that transfection efficiency is lower in *P. chabaudi* than in other rodent malaria models because schizont rupture and re-invasion of mature erythrocytes occurs spontaneously in culture, hindering the collection of large populations of arrested schizonts. Therefore, to improve the efficiency of *P. chabaudi* transfection, we tested whether the reversible inhibitor of the *Plasmodium* PKG - C2 (Collins *et al.*, 2013) - could cause schizonts to arrest and accumulate in culture, thereby increasing the number of viable merozoites available for electroporation.

Addition of C2 at a concentration of 2 µM to the parasite culture led to the accumulation of > 55% mature schizonts (Figure 1A).

Removing C2 (by simply replacing the C2-supplemented media with fresh culture media) allowed normal schizogony to resume and these enriched schizonts could then be electroporated in the presence of plasmids pCAT<(_{250p}>-G6 or pCAT<(_{250p}>-M6 (Figure 1B). Next, electroporated merozoites were transferred into tubes containing uninfected mouse erythrocytes held at 37°C to provide an immediate high-density source of fresh red cells for invasion and ring-formation. This step buys enough time (at least 45 minutes) for multiple transfection procedures to be carried out in the laboratory before the transfectants need to be injected into recipient mice. Significantly, these two protocol modifications improved the efficiency of transfection to such an extent that merozoites could be injected directly into wild-type mice, removing the need for immunodeficient mouse strains for parasite expansion and drug selection. In these ways, we could improve the efficiency, practicability and accessibility of this technique. In our laboratory, we have successfully achieved transformation using pCAT-modified vectors targeting six non-essential loci, with transformed parasites becoming patent at days 9-14 after transfection.

To generate stable, marker-free fluorescent *P. chabaudi* mother-lines, plasmids pCAT<(_{250p}>-G6 and pCAT<(_{250p}>-M6 were transfected into *P. chabaudi* AS parasites according to this modified protocol to introduce GFP and mCherry into the *P. chabaudi* 230p silent locus by double cross-over recombination. After pyrimethamine selection of PcAS-GFP<(_{230p}> and PcAS-mCh<(_{230p}> parasites and genotypic verification (Figure 1C), PcAS-GFP<(_{230p}>-infected mice were provided with 5-FC to select parasites that had lost the drug-selectable cassettes by homologous recombination of *pbdhfr-ts* 3’utr duplicate sequences. The resulting marker-free fluorescent parasites were cloned by limiting dilution to produce the mother-line, PcAS-GFP<(_{ML}>

**Fluorescent PcAS parasites transmit through the mosquito and show wild-type infection dynamics**

PcAS-GFP<(_{ML}>, parasites express GFP strongly throughout the lifecycle and, as with wild-type *P. chabaudi* -AS, undergo schizogony (Figure 2Aii) and re-invasion of erythrocytes in static culture (Figure 2Aiii). PcAS-GFP<(_{ML}>, schizont-infected erythrocytes also display wild-type cytoadhesion properties by forming rosettes with uninfected erythrocytes (Figure 2Aii) and PcAS-GFP<(_{ML}>, parasites efficiently transmit through the mosquito, giving rise to oocysts (Figure 2Bi) and salivary gland sporozoites (Figure 2Biii). Moreover, they establish acute and chronic infections with the same dynamics as wild-type parasites (Spence *et al.*, 2013), and demonstrate attenuated growth after mosquito transmission (Figure 3i). As shown previously (Spence *et al.*, 2013), mice infected with mosquito-transmitted parasites continue to gain weight (Figure 3ii), but experience significant anaemia that cannot be directly attributed to the destruction of infected erythrocytes (Figure 3iii). PcAS-GFP<(_{ML}> parasites can therefore be used to investigate every step of the *Plasmodium* lifecycle.

**PlasmoGEM resources for *P. chabaudi***

We have previously generated large gene deletion and tagging vector libraries for modification of the *P. berghei* genome (Gomes *et al.*, 2015). Scalability relies on robust, parallel engineering of vectors in 96-well plates using the red/ET recombinase system, which in turn requires an arrayed gDNA library, which we
Figure 1. Transfection of *P. chabaudi* AS to create PcAS-GFP<sub>ML</sub>. A) Mature *P. chabaudi* AS schizonts accumulate after culture in the presence of C2 (giemsa-stained smear); infected erythrocytes were smeared 5 minutes after C2 was removed from culture. Merozoites undergoing erythrocyte invasion are arrowed.

B) Schematic representation of the pCAT<sub>230p</sub>-G6 plasmid showing hDHFR and yFcu selectable cassettes, the GFP cassette and 3'PbDHFR-TS direct repeats, allowing recombination and excision of the drug-selectable cassette.

C) Stable integration of GFP into the pc230p locus. To verify correct integration into the pc230p locus in PcAS-GFP<sub>Δ230p</sub> DNA, 1.4kb and 1kb fragments were amplified from the 5' and 3' integration sites using primers 5'intF x 5'intR and 3'intF x 3'intR, respectively. To verify deletion of pc230p, primers 230pF x 230pR were used to amplify a 0.37kb fragment in wild-type but not PcAS-GFP<sub>Δ230p</sub> DNA. To verify the presence of hDHFR, primers dF1 x dF2 were used to amplify a 461bp fragment in PcAS-GFP<sub>Δ230p</sub> but not in wild-type or PcAS-GFP<sub>Δ230p</sub> DNA.

D) Recycling of the drug-selectable cassettes. Following selection with 5-FC, recombined parasites excised the drug-selectable cassettes (insert) and an 830bp fragment was amplified from PcAS-GFP<sub>Δ230p</sub> DNA using primers recF x recR.
**Table 1. Primers.** Restriction enzyme sites are underlined.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<td>5'230pF</td>
<td>atattGCCGGGCATATGTTATAACTAGACCATCG</td>
</tr>
<tr>
<td>5'230pR</td>
<td>ttattACTAGTGTAC1CCATCTGGTAC1C</td>
</tr>
<tr>
<td>3'230pF</td>
<td>atattCTCTGAGAAAATTCCTTTGAGACCCTGAGATG</td>
</tr>
<tr>
<td>3'230pR</td>
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<tr>
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<td>5'intR (230p)</td>
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<tr>
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</tbody>
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**Figure 2.** PcAS-GFP\textsubscript{ML} undergoes schizogony, rupture and rosetting with uninfected erythrocytes in culture and transmit through the mosquito. **A** PcAS-GFP\textsubscript{ML} trophozoites from infected mice were cultured for 4.5 hours in the presence of C2. At 4.5hr and in the presence of C2; i) schizont-infected erythrocytes form rosettes with uninfected erythrocytes; left–hand panel, merged bright and fluorescent fields of live parasites in culture; right–hand panel, fluorescent field. At 4.5hr and after removal of C2; ii) merozoites egress from schizonts and iii) invade erythrocytes (arrowed); merged bright and fluorescent fields. Scale bar = 5μm. **B** i) oocysts in the midgut (day 8), Scale bar = 10μm ii) midgut sporozoites and iii) salivary gland sporozoites (day 14). Scale bar = 5μm.
Figure 3. The virulence of PcAS-GFP<sub>ml</sub> parasites is reset by mosquito transmission and increases after serial passage. C57Bl/6Jax mice were inoculated ip with $1 \times 10^5$ infected red blood cells of PcAS-GFP<sub>mt</sub>, two blood passages from a mosquito-transmitted inoculum (mt-PcAS-GFP<sub>mt</sub>) or with $1 \times 10^5$ infected red blood cells of PcAS-GFP<sub>mt</sub> that had been serially blood-passaged (sbp PcAS-GFP<sub>mt</sub>). Data shown as a pool of n=7 mice for mt PcAS-GFP<sub>mt</sub> and n=8 mice for sbp PcAS-GFP<sub>mt</sub> with mean ± SEM. i) Parasitaemia from tail blood, determined by Giemsa stain and by flow cytometric analysis. ii) Percent weight change from day 0 post infection (p.i.) with mt PcAS-GFP<sub>mt</sub>. iii) Anaemia from day 0 post infection (p.i.) with mt PcAS-GFP<sub>mt</sub> defined using circulating haemoglobin (Hb g/L) levels, determined by tail blood with a Hemocue analyser. Moderate anaemia is defined as 120 g/L and severe anaemia defined as 80 g/L (dotted lines).

constructed in a phage N15-derived linear cloning system with hairpin telomers (Pfander et al., 2011; Zhang et al., 1998). The low copy number of the pJAZZ cloning vector facilitates serial recombinering, and due to its linear nature it can maintain relatively large AT-rich gDNA fragments (Godiska et al., 2010), resulting in targeting vectors with large homology regions and high integration rates (Pfander et al., 2011). Furthermore, since pJAZZ plasmids in our hands are not maintained as episomes when transfected into P. berghei, the background noise in transfection experiments is much reduced, such that within a few days of transfection, barcode counting on a sequencer can be used to phenotype dozens of mutants simultaneously (Bushell et al., 2017; Gomes et al., 2015).

To create PlasmoGEM resources for P. chabaudi, we needed to create such an arrayed library from P. chabaudi genomic DNA. We created a gDNA library in the pJazz-OK Blunt cloning vector, consisting of 7104 clones, of which 5523 could be mapped to version 3 of the P. chabaudi genome (Otto et al., 2014) with an average insert size of 6.7kb (Figure 4A). The cloning efficiency (73%) was similar to our previously generated P. berghei gDNA library resource (Pfander et al., 2011). The library covers at least 50% of the coding-sequence of 4167 protein-coding genes (equivalent to a coverage of 80.7% of all predicted genes in the assembled genome); 3392 (65.7% coverage) genes are covered fully. Using our previously described software, we created automated designs for knock-out vectors for 3665 genes (70.9% coverage) and c-terminal tagging vectors for 3141 genes (60.8% coverage), which are available on our website at https://plasmogem.sanger.ac.uk (Schwach et al., 2015).

Our initial targets for knock-out vectors were genes implicated in signal transduction, and those predicted to encode exported proteins using a number of criteria including existing annotation of gene function, presence of transmembrane domains and ExportPred algorithm scores (Sargeant et al., 2006). Using our vector production pipeline that employ a sequential combination of recombinase mediated engineering (recombineering) and Gateway<sup>TM</sup> technology (Pfander et al., 2011), (Figure 4B)), we produced a first batch of 406 (7.9% coverage) barcoded
Figure 4. Generation of PlasmoGEM resources for genetic modification of P. chabaudi. 

A) Insert-size distribution for P. chabaudi genomic DNA (gDNA) clone library showing number of clones for each genomic insert-size category, with an average genomic clone size of 6–7 kb.

B) The pJAZZ OK Blunt vector (i) was used to clone size-selected P. chabaudi genomic DNA fragments to generate the PcG01 and PcG02 genomic library clones (ii). The pJAZZ-OK Blunt vector encode hairpin telomers (shown in black), a telomerase gene (TelN), replication factor and origin (repA), regulator of replication (cB) and a kanamycin resistance gene (kanR). Genomic library clones are converted into gene targeting vectors by a two-step process. Firstly, recombineering with a PCR amplicon carrying the dual bacterial selectable marker zeo-pheS (zeo-pheS) flanked by 50 bp homologous targeting sequences (black box) introduces the intended sequence edit (iii). Subsequently, LR-Gateway cloning between AttR and AttL sites (grey box) with the pR6K attL1-3xHA-hdhfr-yfcu-attL2 plasmid, facilitates the exchange of the zeo-pheS cassette for the parasite positive-negative selection marker human dihydrofolate reductase-uridyl phosphoribosyl transferase (hdhfr-yfcu), (iv). Linear transfection-ready vectors are prepared for transfection by NotI digest and integrate with high efficiency into the P. chabaudi genome at the gene of interest (GOI) locus (v).
single-gene knock-out vectors that passed quality control by full-length sequencing. The vectors are available to request as part of the PlasmoGEM project.

High efficiency transformation of wild-type and PcAS-GFP<sub>ml</sub> P. chabaudi using PlasmoGEM vectors

To compare the efficiency of transfection between modified pCAT plasmids and pJAZZ-derived PlasmoGEM long homology-arm gene disruption vectors, we targeted loci encoding p230p and genes encoding members of the P. chabaudi Cysteine Repeat Modular Protein (PcCRMP) family, which are not essential for blood-stage replication (Thompson et al., 2007), in wild-type P. chabaudi AS. As an example, the strategy used to modify the PCHAS_081270 locus, encoding PcCRMP1, is shown in Figure 5. Patent parasitaemia was observed in pJAZZ vector-transfected lines on day 8–9 following infection and in pCAT vector-transfected lines on day 10–14 (Figure 5C), suggesting that pJAZZ vectors confer > 64-fold higher transfection efficiency (assuming P. chabaudi AS has an 8-fold proliferation rate/cycle).

Figure 5. Modification of non-essential loci with pCAT and pJazz plasmids. A) Left hand panel (LHP): schematic representation of the pCAT<sub>crmp1</sub>-M6 plasmid and the pcrcrm1 locus after transfection. Right hand panel (RHP): to verify correct integration into the pcrcrm1 locus, 1.22kb and 1.17kb fragments were amplified from the 5' and 3' integration sites using primers 5'intF.crmp1 x 5'intR.crmp1 and 3'intF.crmp1 x 3'intR.crmp1, respectively. To verify deletion of pcrcrm1, primers crmp1F x crmp1R were used to amplify a 306bp fragment in wild-type but not PcAS-mCh<sub>Δcrmp1</sub> DNA. To verify the presence of hdhfr, primers dF1 x dF2 were used to amplify a 461bp fragment in PcAS-mCh<sub>Δcrmp1</sub> but not in wt DNA. B) LHP: schematic representation of the PGEM-600068 construct and the pcrcrm1 locus after transfection. To verify correct integration into the pcrcrm1 locus, a 2.9kb fragment was amplified from the 3' integration site using primers GW1 x GT1.crmp1. To verify deletion of pcrcrm1, primers crmp1F2 x crmp1R2 were used to amplify a 627bp fragment in wt but not PcAS-mCh<sub>Δcrmp1</sub> DNA. To verify the presence of hdhfr, primers dF1 x dF2 were used to amplify a 461bp fragment in PcAS-mCh<sub>Δcrmp1</sub> DNA. C) Day of patent parasitaemia following transfection with pCAT plasmids (n=9) and pJazz constructs (n=10) targeting non-essential loci. Parasitaemia was monitored by giemsa stain from days 7–16 following transfection. Patency was determined when parasitaemia was at least 0.005%. D) LHP: schematic representation of the PGEM-610764 construct and the pcrcrm4 locus after transfection into P. chabaudi<sub>AS</sub> wild-type and PcAS-GFP<sub>ml</sub> parasites. To verify correct integration into the pcrcrm4 locus, a 3.3kb fragment was amplified from the 3' integration site using primers GW1 x GT1.crmp4. To verify deletion of pcrcrm4 in wild-type and PcAS-GFP<sub>ml</sub> parasites, primers crmp4F x crmp4R were used to amplify a 700bp fragment in wild-type but not PcAS-Δcrmp1 DNA. To verify the presence of hdhfr, primers dF1 x dF2 were used to amplify a 461bp fragment in PcAS-Δcrmp4 but not in wild-type DNA.
To next compare the transfection efficiency of PGEM vectors in wild-type and PcAS-GFP(+) parasites, we transfected a construct targeting PcCRMP4 (PCHAS_1304000; Figure 5D). PcCRMP4-deleter parasites were patent by day 9 post-transfection in both parasite lines and showed integration into the pccercmp4 locus and deletion of the gene. Thus, we have demonstrated the generation of marker-free fluorescent mother-lines that can now be genetically-modified using the high-efficiency vectors, available from PlasmoGEM. This enhanced toolbox is now ready to kick-start Plasmodium chabaudi genetic studies.

**Summary**

The improvements in the efficiency, practicability and accessibility of *P. chabaudi* transfection techniques reported here have enabled us to reproducibly generate a series of fluorescently-tagged and gene-deletion parasite lines. Transfections were carried out using transfection vectors derived from the versatile pBAT series of plasmids (Kooij et al., 2012) that can rapidly be adapted for use in gene targeting and tagging strategies, and we have used these to generate fluorescent lines of distinct *P. chabaudi* genotypes (unpublished report). These plasmids also allow recycling of the positive drug-selectable marker and, therefore, sequential genetic modifications. For *P. chabaudi*, this feature may be particularly useful for analysis of the function of multigene families that are not individually essential for blood-stage replication but have been proposed to play a role in immune evasion and modulation, including members of the Plasmodium Cysteine Repeat Modular Protein (PCRMP) family (Douradinha et al., 2011; Thompson et al., 2007). Thus, we can now study the function of the multi-gene families during both acute infection and chronicity in the face of the host immune response, and we can explore whether family members have overlapping and compensatory roles by performing double and multiple gene deletions, making use of our ability to recycle the selection marker using negative selection.

We have generated a genome-scale *P. chabaudi* genomic library that can effectively be converted into PlasmoGEM linear, long genomic library (Kooij et al., 2012). Genomic loci can be cloned using this library, and we have used these to generate fluorescent lines of distinct *P. chabaudi* genotypes (unpublished report). These plasmids also allow recycling of the positive drug-selectable marker and, therefore, sequential genetic modifications. For *P. chabaudi*, this feature may be particularly useful for analysis of the function of multigene families that are not individually essential for blood-stage replication but have been proposed to play a role in immune evasion and modulation, including members of the Plasmodium Cysteine Repeat Modular Protein (PCRMP) family (Douradinha et al., 2011; Thompson et al., 2007). Thus, we can now study the function of the multi-gene families during both acute infection and chronicity in the face of the host immune response, and we can explore whether family members have overlapping and compensatory roles by performing double and multiple gene deletions, making use of our ability to recycle the selection marker using negative selection.

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**Data availability**

Underlying data

PlasmoGEM vector sequence and design data are available as a public resource at https://plasmogem.sanger.ac.uk.

Open Science Framework: Marr et al., An enhanced toolkit for the generation of knockout and marker-free fluorescent Plasmodium chabaudi. https://doi.org/10.17605/OSF.IO/65RW3

This project contains the following underlying data:

- Original gel and microscopy images for Figure 1, Figure 2 and Figure 5 in TIF/TIFF format
- pCAT plasmid sequences in DOCX format
- Raw parasitaemia, weight change and anaemia data underlying Figure 3 in XLSX format
- Raw data underlying Figure 5C in XLSX format (Tfn_pCATpJAZZ)
- Gating strategy for peripheral parasitemia in PPTX format

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

**Acknowledgements**

We thank Dr Taco Kooij for the kind gift of pBAT-sil6 plasmids and the European Malaria Reagents Repository for *P. chabaudi* AS.

**References**


Douradinha B, Augustijn KD, Moore SG, et al.: Plasmodium Cysteine Repeat Modular Proteins 3 and 4 are essential for malaria parasite transmission from...
the mosquito to the host. Malar J. 2011; 10: 71.

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Malaria in humans is caused by more than 7 different species of protozoan parasite within the *Plasmodium* genus, with *Plasmodium falciparum* causing the majority of disease and death. Studies into the biology of malaria parasites primarily rely on the use of a few species of human malaria parasites that can be cultured in vitro and 3 different species of rodent malaria parasites which can be maintained in mice in the lab. The functions of a majority of proteins are conserved across all species, so the use of multiple species allows us to make use of key experimental advantages of each system to uncover shared biology. It also offers the opportunity to compare and contrast results to study key differences between the species. Whilst the transfection technology for the rodent malaria parasite *P. chabaudi* has lagged behind other species, it none the less has proved invaluable in the study of key aspects of host-parasite interactions. This work addresses a key current drawback in the *P. chabaudi* model with the presentation of 3 significant advancements in experimental genetics approaches – optimisation of transfection efficiency, generation of marker-less transgenic lines and generation, and testing of a *P. chabaudi* plasmoGEM construct library. The work provides a major technical advancement for the model and provides an important new resource for the field.

The comments are all minor but I hope should provide some small improvements to the paper.

**General comments:**

- “two mice to provide infected blood for transfection and eight mice as recipients of parasites transfected with four plasmids in replicate” Why were two mice used for each plasmid per transfection? For replicates, it would be more appropriate to carry these out across different parasite preparations/transfections which are likely the biggest variable for transfection efficiency.

- “a custom software pipeline as previously described for generating a *P. berghei* resource” Whilst the reference describes general parameters an additional direct link to the software and source code (journal requirement) should be added. The version number of software used should also be made clear, I imagine it may be different between the two papers.
• Images in fig1 (smears) are very low quality. I don’t dispute the interpretation but it is very difficult to see the condition of the parasites in that resolution.

• “we tested whether the reversible inhibitor of the Plasmodium PKG - C2 (Collins et al., 2013)” is this the right reference? C2 is not used for synchronisation in this paper. Perhaps it should be https://doi.org/10.1371/journal.ppat.1003344 (which has the same first author and year).

• C2 has been used in P. knowlesi for transfections for similar reasons. Here viable was found to decrease if held beyond 3 hours. Could the authors comment on the incubation time used (4.5 h) and whether loss of viability was detected after longer incubations? This will help guide correct usage by other labs.

• “undergo schizogony and re-invasion of erythrocytes in static culture (Figure 2A)” to what extent can P. chabaudi be cultured longer term? Can you provide an indicator as to relative growth rate in vitro, or whether it can be maintained for multiple cycles? If this has been demonstrated previously could this reference be added?

• Figure 2 title seems to relate to just panel A, the title could be changed to include a reference to transmission states and A) moved to the second sentence. Scale bars are only indicated in one image each for A/B i, ii, iii, but the images are at different magnification. The sporozoite images appear to be at a much greater magnification so need a separate scale bar. The data from Figure 3 are also duplicated in the figure image.

• “terminal tagging vectors for 3141 genes (60.8% coverage), which are available on our website at https://plasmogem.sanger.ac” hyperlink truncated.

• Summary - I would additionally suggest some discussion of where these technologies fit into the wider scheme of those used in other systems. Combining the optimisation of transfection with Cas9 based approaches, and the huge vector resource available through PlasmoGEM could create a formidable system. Also highlight one of the current downsides of the PlasmoGEM system which is that the markers cannot be recycled.

• Summary - In vitro reinvasion is mentioned, could short term culture be used for transfection recovery to reduce animal use?

References

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes
Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Malaria parasite experimental genetics (P. berghei, P. falciparum and P. knowlesi), malaria parasite biology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

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**Author Response 08 Jun 2020**

**joanne Thompson**, University of Edinburgh, Ashworth Laboratories, The King's Buildings, Edinburgh, UK

Thank you for your fast review and for your interesting and insightful comments. We have made the minor textual revisions you have suggested in version 2 of the manuscript and have responded to your specific points inline below;

- two mice to provide infected blood for transfection and eight mice as recipients of parasites transfected with four plasmids in replicate" Why were two mice used for each plasmid per transfection? For replicates, it would be more appropriate to carry these out across different parasite preparations/transfections which are likely the biggest variable for transfection efficiency.

**Response:** Two mice were infected with replicate transfection reactions for each plasmid to ensure that replicates of the modified lines generated originated from independently-transfected merozoites (rather than through amplification of a single transfectant). We have now up-dated Fig 5C to show that a sufficient number of transfection experiments were carried out, using different parasite preparations/transfections which are likely the biggest variable for transfection efficiency.

- a custom software pipeline as previously described for generating a *P. berghei* resource" Whilst the reference describes general parameters an additional direct link to the software and source code (journal requirement) should be added. The version number of software used should also be made clear, I imagine it may be different between the two papers.

**Response:** The vector designs are not generated by a single piece of software, instead a collection of scripts to automate different tasks. These would require substantial effort before they could be distributed for use by others. However, the rules for vector designs were as previously described and the output of the software pipeline are the gene modification designs, which are fully disclosed and available for scrutiny at [https://plasmogem.sanger.ac.uk/](https://plasmogem.sanger.ac.uk/) by reviewers and readers.
Furthermore, the software pipeline is not essential in the design of gene modification vectors. We could have designed the same constructs manually, using similar design guidelines as used by the software.

- Images in fig1 (smears) are very low quality. I don’t dispute the interpretation but it is very difficult to see the condition of the parasites in that resolution.

Response: These images are selected to show that segmented schizonts, containing up to 8 merozoites form in culture. Images of the whole field of view can be seen in the original data file available at https://doi.org/10.17605/OSF.IO/65RW3.

- C2 has been used in P. knowlesi for transfections for similar reasons. Here viable was found to decrease if held beyond 3 hours. Could the authors comment on the incubation time used (4.5 h) and whether loss of viability was detected after longer incubations? This will help guide correct usage by other labs.

Response: The parasites are actually cultured in the presence of C2 for 2.5hrs; C2 is added to the culture, together with fresh media, 2 hrs after the culture is set up (see methods). David Baker had advised that there might be costs to viability with long-term culture, although I should note that the aim here is to synchronise developing schizonts, rather than to arrest schizonts for any period of time. We haven’t tested longer incubation times, so I’m afraid we cannot comment further on this.

- “undergo schizogony and re-invasion of erythrocytes in static culture (Figure 2A)” to what extent can P. chabaudi be cultured longer term? Can you provide an indicator as to relative growth rate in vitro, or whether it can be maintained for multiple cycles? If this has been demonstrated previously could this reference be added?

Response: It has not been our aim to develop a long-term culture system for P. chabaudi so we cannot comment formally on the relative growth-rate in culture or maintenance for multiple cycles. On the small number of occasions that we have cultured schizonts with fresh erythrocytes, we have observed re-invasion and development to the young trophozoite stage, but we have not attempted to optimise this procedure or to ‘culture-adapt’ parasite lines.

- Summary - I would additionally suggest some discussion of where these technologies fit into the wider scheme of those used in other systems. Combining the optimisation of transfection with Cas9 based approaches, and the huge vector resource available through PlasmoGEM could create a formidable system. Also highlight one of the current downsides of the PlasmoGEM system which is that the markers cannot be recycled.

Response: The hdhfr-yfcu marker used in P. berghei vector resource and the P. chabaudi vectors is recyclable since the hdfr-yfcu cassette is flanked by duplicated pdbhfr 3’utr sequences, which facilitates efficient recombination under 5-FC pressure. It is referenced in the original methods publication by Pfander et al 2011 (Supplementary Figure 1. Map of plasmid pR6K attL1-3xHA-hdhfr-yfcu-attL2).

- Summary - In vitro reinvasion is mentioned, could short term culture be used for transfection recovery to reduce animal use?

As described above, we have not optimised techniques for long-term in-vitro culture of P. chabaudi parasites, so cannot perform drug-selection and amplification of the parasites without growth in vivo.

**Competing Interests:** No competing interests were disclosed.
This paper describes the generation of a new fluorescent \textit{P. chabaudi} mother line that can be used to knockout and tag genes using the \textit{P. chabaudi} specific PlasmoGEM vectors that have also been created as part of this study. Together this line and the PlasmoGEM vectors will facilitate the study of gene function in \textit{P. chabaudi}, a rodent species that causes a chronic infection with periodic recrudescence as a result of its antigenic variation. Hence the ability to genetically manipulate this rodent malaria species will be of value to malaria researchers.

The methodologies were thoroughly described, the results are robust and conclusions valid. My comments really relate to improving the manuscript for clarity.

1. I found the methodology described in the second paragraph of the 'Design and generation of PlasmoGEM gene targeting vectors for \textit{P. chabaudi}' somewhat confusing and whilst this is schematically presented in Figure 4 it would be good to label (i), (ii), etc and for example indicate which is the pR6K attL1-3xHA-hdhfr/yfcu-attL2 plasmid so that the steps described in the methods can be readily followed in the figure. Also for this figure legend, reword so that mention of (i) comes before (ii). Also show where NotI cuts in the figure.

2. PCR step in 'Transfection and selection of fluorescent \textit{P. chabaudi} parasites' - it looks like the annealing step has been omitted.

3. Figure 1: include the primers used for the PCRs underneath the figure for greater clarity. E.g. presumably in Fig 1B df/dr was used in first panel and recF and recR in second?

4. Table 1: no restriction sites have been underlined.

5. Results: Design of transfection vectors: one has to go to the methodologies to see what pCAT230p-G6 and pCAT230p-M6 are. Indicate they are modified versions of the pSIL6 vector that incorporates...

6. Figure 2. Panel 2Ai - it is hard to tell if these are rosettes or if there is just a high density of cells on the slide. There is no reference to figure 2ii in the text. Figure 2 also contains figure 3 - please delete.

7. Figure 3: Panel B and C are not described in the text at all. How do these compare to blood stage parasites? Best if they are also on the same graph. Please confirm that the mice increase in weight after infection rather than decrease.

8. Figure 5: Again it would be best to show the oligo combinations used in the PCR beneath the figure rather than in the figure legend for greater clarity. Panel C would be better as a scatter plot to show variability - is the difference also significant?
9. Minor errors:
   keywords - Transfection should be lower-case t.
   c-terminal tagging - The c should be in capitals.
   gel electrophoresis In 'Transfection' in methodology, there should be a space between gel and electrophoresis.

Is the rationale for developing the new method (or application) clearly explained?
Yes

Is the description of the method technically sound?
Yes

Are sufficient details provided to allow replication of the method development and its use by others?
Yes

If any results are presented, are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions about the method and its performance adequately supported by the findings presented in the article?
Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** molecular biology, malaria transfection, animal studies

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

---

**Author Response 08 Jun 2020**

**joanne Thompson**, University of Edinburgh, Ashworth Laboratories, The King's Buildings, Edinburgh, UK

Thank you for your fast response and for the very interesting and constructive comments you have made. We have made the minor changes you have requested in the text and figure annotation in version 2 of the manuscript and have replied to specific points in the text below.

>6. Figure 2. Panel 2Ai - it is hard to tell if these are rosettes or if there is just a high density of cells on the slide.

To clarify, the images shown are of live parasites in culture; a comment has been included in the figure legend to explain this. Since they are live, there is a lot of movement of cells in the cultures, and only cells that are tightly adherent remain together. This is only observed for schizont-infected erythrocytes, and erythrocytes infected with all other stages do not form rosettes (please refer to original data for more images of this, available at [https://doi.org/10.17605/OSF.IO/65RW3](https://doi.org/10.17605/OSF.IO/65RW3)). Interestingly, we have observed that the percentage of schizonts forming rosettes varies
between *P. chabaudi* strains and decreases after serial blood-passage.

>7. How do these compare to blood stage parasites? Best if they are also on the same graph.

The kinetics of parasitaemia and infection of wt *P. chabaudi* AS parasites have been extremely well documented, including in the Spence et al (2013) paper cited here. These publications have demonstrated that, although there may be some variation (likely caused by a number of factors, including mouse housing conditions and number of times the parasites have been serially blood-passaged since mosquito-transmission), serially blood passaged *P. chabaudi* parasites reach a peak parasitaemia of ~20% between days 7-9 of infection, and are then resolved, whilst recently mosquito-transmitted parasitaemia are attenuated, reaching a peak parasitaemia of <5%. Since we have demonstrated that the PcAS-GFP<sub>ml</sub> parasites used in this study show the same parasitaemia kinetics and virulence, we did not believe that it was justified to increase our animal usage by carrying out concurrent wt infections. We have therefore described the wt infection dynamics in the text and cited the relevant previous reports.

**Competing Interests:** No competing interests were disclosed.