# Introduction

Malaria is an important vector-borne disease of public health significance, which is caused by one or a combination of *Plasmodium* parasites. There are several species of *Plasmodium* parasites that are known to cause human disease, of which *P. falciparum* causes the most severe clinical outcomes and is responsible for the majority of infections in Africa (1–3). Humans are infected with malaria through the bite of a female *Anopheles* mosquito, which injects the *Plasmodium* parasites into the bloodstream (Figure 1). Once injected into the bloodstream, sporozoites migrate to and differentiate in liver cells, forming schizonts. These rupture out of liver cells and infect erythrocytes, resulting in the blood stages. Blood stage parasites are asexual forms which are responsible for clinical disease. They undergo asexual reproduction in erythrocytes, finally rupturing the erythrocyte to be released into the blood to infect more cells. Some asexual parasites differentiate into sexual forms (gametocytes), which are ingested by a mosquito during a blood meal. Therefore, it is the sexual forms of the parasite that are responsible for human-to-mosquito transmission.

Worldwide there were an estimated 229 million malaria cases and 409,000 deaths in 2019 (4). The overwhelming majority of these occurred in African children under the age of 5 years. Not only are children under 5 years disproportionately affected clinically but their epidemiology is distinct, with malaria parasite density being significantly higher (5–9). This is attributed to ongoing loss of maternal immunity with increasing age and a lack of acquired immunity due to reduced exposure time (7). Even though there has been a reduction in malaria incidence per 1,000 population at risk in the African region over the past 20 years, overall cases have been increasing, due to a rapidly accumulating population, putting an increasing strain on healthcare systems (3).

Nigeria has more malaria than any other country, with 27% of all cases and 23% of all deaths worldwide in 2019 (3). Malaria is endemic countrywide and around 80% of the population live in high transmission areas (4), with substantial morbidity burden in all zones (11). The World Health Organisation (WHO), have instigated a ‘High burden to high impact’ approach to tackling malaria, to provide a ‘wake-up call’ to countries with the highest burden, which is especially pertinent to Nigeria, where there is estimated to be an increasing number of cases annually (4,12). There is a high level of heterogeneity in regards to the ecology and malaria transmission in the country, which may be greater than seen in smaller and more intensively studied counties such as the Gambia (13).

To be able to understand why there is heterogeneity in malaria burden and to be able to strive for future reductions, an in-depth understanding of the local epidemiology is crucial (14). Malaria parasite prevalence is an aspect of the epidemiology that is often focused on but there is a need to widen our understanding to include other possibly informative parameters, one such could be parasite density data. Parasite density data has predominantly previously been used to examine factors determining disease severity, been discussed in relation to sub-microscopic infections and additionally has been a secondary consideration in intervention trials (15–18). There have previously been small-scale epidemiological investigations in a single location regarding parasite density in Nigeria (7,19)**,** and in other African countries (8,20), in which the majority focus on *P. falciparum*.

Understanding how parasite density data can be used to inform us about changes in endemicity has not been investigated previously on a national scale and is the reason why this research is required. Endemicity is defined as the level of transmission occurring and as a proxy to denote malaria prevalence, which is influenced by a complex interplay between anthropogenic factors, the parasite, vector and environment (21). There are numerous methods by which endemicity can be quantified such as spleen rate, parasite rate, annual parasite incidence and entomological inoculation rate (22). Against the backdrop of stalling malaria reductions, insecticide resistance and parasite evolution, this project seeks to address the lack of research connecting malaria prevalence and density, possibly providing a new method to understand levels of endemicity (23). Furthermore, finding novel methods to assess endemicity are required due to the need for more targeted and efficient control programs.

Through three nationwide surveys, it has been shown that in Nigeria the prevalence of malaria has been decreasing from 42% to 27% to 23% in 2010, 2015 and 2018 respectively (24–26). Overall, the geopolitical zones in the north contained a higher prevalence than the southern zones (25,26)**.** Looking into these surveys in more detail, revealed that malaria infections in children under 5 years had reduced in most Nigerian states (27). However, there were differences in the timing and magnitude of reductions in different geographic locations. Between 2010 and 2018, there were significant reductions in malaria prevalence in just under 70% of the states in Nigeria. This is highlighted by the fact that in 2010, 17 states had a prevalence in excess of 40% but in 2018 this had reduced to only one state. However, between 2015 and 2018 many reductions in the prevalence in the southern states had stopped or been reversed. This project will utilise the data from the three nationwide surveys but instead of focusing on malaria parasite prevalence exclusively, it will investigate changes in malaria parasite density in conjunction with prevalence. Additionally, the sexual prevalence/density and *Plasmodium* species data, which has not previously been examined, will be investigated in connection with the asexual density data.

Although there is evidence to show that malaria prevalence is decreasing in Nigeria, this may not translate into reducing parasite densities. Immunity plays a significant role in reducing parasite densities and so you may expect to see more low-density infections in highly-endemic areas (28). In line with this theory, as malaria endemicity decreases, it has been shown that acquired immunity wanes (13,29) and so when an infection does occur, the parasite density may not be supressed leading to increased high-density infections. On the other hand, there is reason to expect that at the community level when malaria becomes less endemic you may see more carriage of low-density infections (30)**.** This could occur as a result of historic infections containing a lower density and these being more common when endemicity decreases (31).

A historic but keystone reference which previously investigated malaria epidemiology in Nigeria is the Garki project (32). This project was a longitudinal study between 1969-1976 looking at the effects of different malaria control interventions, with continued epidemiological investigation of both treated and untreated villages, allowing a protracted view of the epidemiological situation. Parasitological investigations in the absence of any control intervention showed that infants (children aged <1 years) had a reduced parasite density distribution as compared to children aged 1-4 years. Additionally, a sex differentiation was found in that males over the age of 5 years had consistently higher parasite prevalence and density, however, before 5 years of age there was no differentiation. Parasitological findings during control activities (with propoxur) showed that overall infections had a reduced density of asexual stages, however, this intervention did not affect the sexual density. In comparing sprayed and unsprayed villages, in children aged 1-4 years, sprayed villages had a lower density of *P. falciparum*. However, there were parasitological variations in comparably treated villages that were unrelated to the coverage of interventions. This shows that even with the same application of control interventions, different epidemiological outcomes can occur, evidencing the complexity of the infection dynamics.

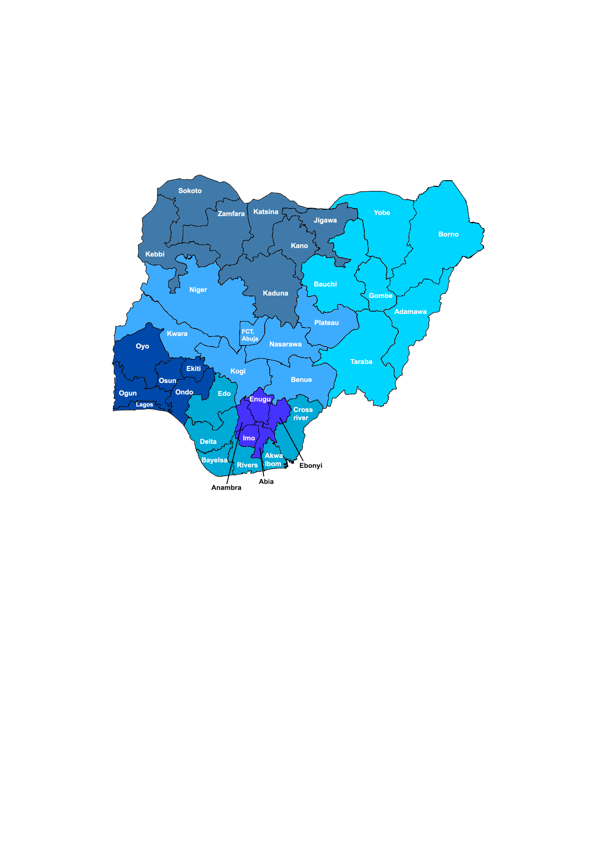
Malaria infections can be the result of one *Plasmodium* species but can also occur as a result of multiple species, known as co-infections. The dynamics of co-infections have been investigated previously with conflicting results, and so species data will additionally be investigated to understand if densities change based on the species present. The Garki project previously investigated *Plasmodium* species differences, finding that *P. falciparum* infections most commonly caused long duration patent parasitaemia (32). This was found to be a result of a high conversion rate and concurrently low clearance rate. *P. malaria* was found to have generally lower conversion and higher clearance rates than *P. falciparum*, with *P. ovale* having the lowest and highest respectively. This meant that episodes of patent parasitaemia with *P. ovale* were the least common and of the shortest duration. Looking at co-infections in untreated villages it was found that the chance of acquiring or maintaining an infection with one species was increased if another species was already present. This relationship was amplified in villages sprayed with propoxur, where *P. falciparum* and *P. malariae* co-infections were seen in excess. Therefore, this shows evidence for an increase in co-infections as a result of control measures.

# Methods

## Study area, blood sample collection and parasitaemia quantification

Nigeria is a country in Africa, which is comprised of 6 geopolitical zones and 36 states (Figure 2). It has a land area of 923,768 km2 and is the most populous country in Africa, with an estimated population growth of around 3% per annum. Currently the population is estimated to be 211 million and the United Nations project that this could reach 401 million by 2050 (33). Malaria is endemic in all regions of Nigeria, with sustained transmission occurring in diverse ecosystems such as mangrove swamps, semi-arid savannas, freshwater swamp forests and lowland rainforests.

Climatic conditions are characterised by extensive humidity and rainfall, being tropical in nature in the south and savannah in the north. The dry season occurs from November to March and the wet season from April to October. In the North (savannah areas), peak rainfall occurs from August to September, however, in the south (forest areas) there are two peaks of rainfall in July and September. Regarding the rainfall distribution annually, more is seen in the south than the north.



**North East**

**North West**

**North Central**

**South West**

**South South**

**South East**

**Figure 2: Map of Nigeria**. Nigeria is located on the Western coast of Africa, neighbouring Benin, Niger, Chad and Cameroon. The 6 geopolitical zones and individual states are named on the map.

Children aged 5-59 months (under 5 years) had capillary blood finger prick samples collected for all surveys. Thick peripheral blood films were prepared on uniquely barcoded slides. These slide were transferred to an accredited reputable research facility with a gold-standard microscopy diagnostic centre (34). On receipt, each slide was scanned into an electronic database. Using the WHO-recommended malaria microscopy standard operating procedure (MM-SOP-09), each slide was stained with 3% Giemsa (35).

Each slide reading was conducted independently by two WHO-certified grade 1 microscopists, where 200 fields were analysed, with both asexual and sexual parasites and white cells counted. Additionally, microscopy was utilised for species identification using morphology. A negative result was recorded if no parasites were seen after the inspection of 200 microscopy fields. The process was reviewed by a slide coordinator who checked for concordance. If discordance was noted, another WHO-certified grade 1 microscopist conducted an independent read. A final consensus asexual/sexual parasite density reading was produced based on the mean of the two independent results. After counting, the asexual/sexual parasite density was calculated based on individuals containing an estimated average white blood cell (WBC) count of 8000/μL using the following formula:

**Parasites / μL blood =**

## Statistical analysis

Asexual/sexual prevalence was determined by positive samples over the total number of samples tested and prevalence analysis was conducted based on Pearson’s Chi squared test. Changes over time in parasite density in each of the 36 states and the six major geopolitical zones was assessed through calculating the geometric mean parasite density, 95% confidence intervals, and conducting statistical tests of differences in the distributions between states and regions. The malaria parasite densities were statistically analysed based on the non-parametric Mann-Whitney test for binary exposures and Kruskal-Wallis test for more than two exposure levels. If the Kruskal-Wallis test found an overall significant difference, then pairwise comparisons using the Wilcoxon rank sum test with continuity correction were conducted.

This project aimed to understand the additional benefit of looking at malaria parasite density data, on top of the information provided by prevalence data. To be able to rigorously achieve this, information provided by the prevalence data needed to be excluded. Therefore, for the majority of the study, individuals negative for parasites were excluded from the analysis. If analysis occurred based on all individuals (positive and negative) then the variation in the density would largely and mainly be driven by differences in prevalence. Therefore, only looking at the density distribution in infected individuals meant that the analysis was not overshadowed by prevalence differences and the additional benefit of density data could be determined. Where analysis was based on all individuals (positive and negative) this is stated.

Asexual densities were recoded into three categories (< 1000 parasites/μL of blood, 1000–9999 parasites/μL of blood and ≥ 10,000 parasites/μL of blood), with analysis based on Pearson’s Chi squared test. Spearman’s non-parametric rank correlation tests were performed, for example, examining the association between asexual parasite prevalence and density across the states for each survey. Data from the Federal Capital Territory, Abuja was excluded from each correlation analysis as it could not be representatively sampled. Statistical analyses were performed using STATA 16.1 and R 4.1.0.

## 3.4. Ethical considerations

All the data was produced under the Demographic and Health Surveys (DHS) Programme (https://dhsprogram.com/) from national population surveys. The data was anonymised, being provided by the UNICEF Multiple Indicator Cluster Surveys (MICS) team (http://mics.unicef.org/surveys). Procedures and questionnaires for the surveys were reviewed and approved by the ICF Institutional Review Board (IRB), and by the National Health Research Ethics Committee of Nigeria (NHREC).

# Results

## Study population

A total of 7783 Nigerian children under 5 years of age participated in the study. Overall, the mean age was 2.4 years (SD = 1.4) and 49.0% were females (3812/7783). Basic study characteristics are presented in Table 1.

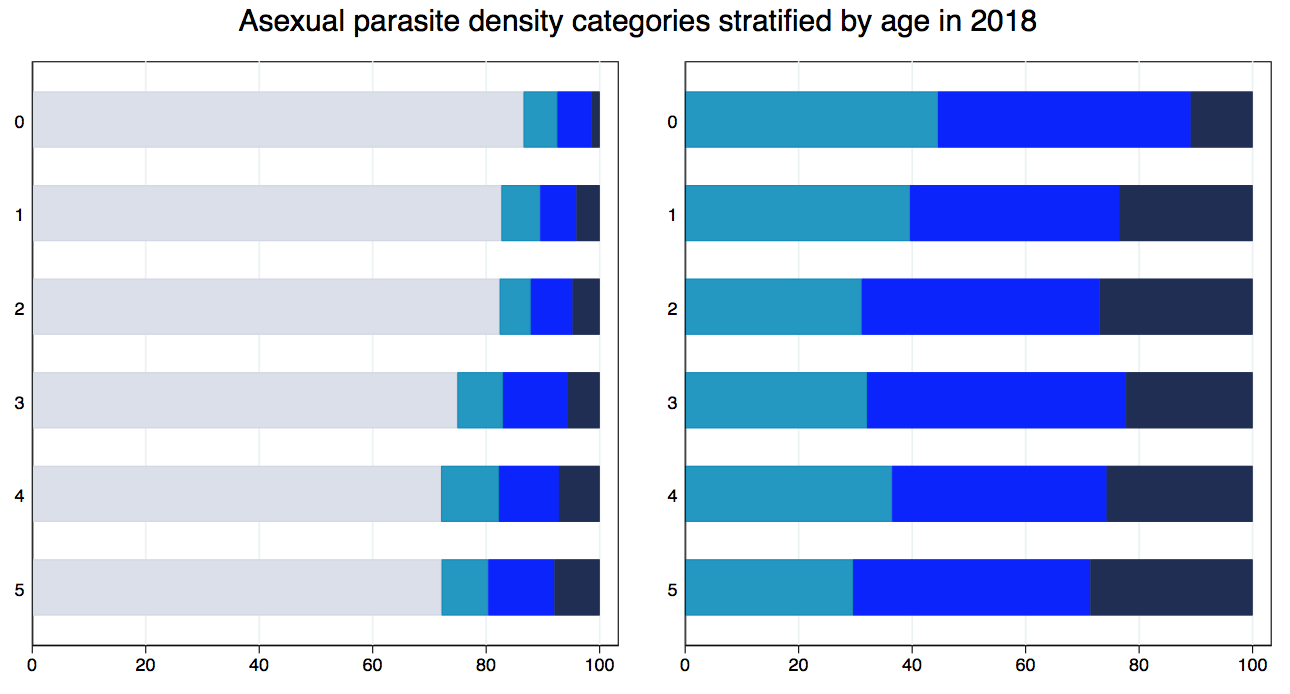
|  |  |
| --- | --- |
|  | **2018**  (N=7,783) \* |
| **Age (years), % (n)** |  |
| 0 | 9.7 (754) |
| 1 | 21.0 (1,637) |
| 2 | 21.0 (1,638) |
| 3 | 22.5 (1,752) |
| 4 | 21.2 (1,653) |
| 5 | 4.5 (349) |
| **Gender, % (n)** |  |
| Female | 49.0 (3,812) |
| **Geopolitical zone, % (n)** |  |
| North-Central | 17.6 (1,368) |
| North-East | 17.9 (1,391) |
| North-West | 23.1 (1,797) |
| South-East | 15.5 (1,207) |
| South-South | 10.9 (852) |
| South-West | 15.0 (1,168) |

### Asexual parasite density

Asexual parasite prevalence was 21.5% (1675/7783) and the asexual parasite densities ranged from 15 to 485,609 parasites/μL, with the geometric mean being 2242 parasites/μL (95% CI = 2032-2473 parasites/μL). Asexual parasite densities did not differ in relation to sex (geometric mean: 2187 parasites/μL, 95% CI: 1909-2505 parasites/μL for males and 2304 parasites/μL, 95% CI: 1998-2657 parasites/μL for females, p-value=0.5).

When including all children in the analysis (those positive or negative for parasites), the proportion with ≥ 10,000 parasites/μL increased with increasing age, with 1.6% in children under 1 years, 4.2% in 1-year olds, 4.8% in 2-year olds, 5.8% in 3-year olds, 7.3% in 4-year olds and 8.0% in 5-year olds (**Figure 4a**). Conversely, the proportion with no infection detected by microscopy decreased with increasing age, with 86.7% in 0-year olds, 82.6% in 1-year olds, 82.3% in 2-year olds, 74.8% in 3-year olds, 72.1% in 4-year olds, 71.9% in 5-year olds (**Figure 4a**). There was strong evidence for a difference between asexual category and age (p<0.001).

When only infected children were included in the analysis, a different pattern emerged (**Figure 4b**). The percentage with ≥ 10,000 parasites/μL was similar in all age groups (between 23.1-28.6%) apart from the in the youngest age group (12.0%). A significant difference between asexual category and age was found (p=0.02), however, this was less significant than when all individuals were included in the analysis.



**Percent (%)**

**Age (years)**

**A**

**B**

Chart

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**Figure 4: Asexual parasite density categories stratified by age in 2018,** highlighting that different information can be understood from analysis based on only positive individuals (B) as compared to when all individuals are included in the analysis (A).

When analysing the geometric mean density by age, a consistent finding to the categorical analysis above was observed, where the youngest age group had the lowest geometric mean (1414 parasites/μL, 95% CI: 985-2030 parasites/μL)(Figure 5). It then increased to a maximum of 2750 parasites/μL (95%CI: 2161-3499) at 2 years of age. After 2 years of age, the asexual parasite density decreased and then increased slightly. There was evidence for a difference between asexual parasite density between age groups (p=0.04). Through conducting pairwise comparisons, this difference was shown to be driven through the 0-year age group being significantly different to the rest (Appendix 1).

Geopolitical zone asexual density differences were investigated on the premise of the zones containing differences in the prevalence of malaria, with the north generally being higher than the south (21). Regarding the distribution of parasitaemia by geopolitical zone in 2018, the highest density was recorded in the North-West (geometric mean 2650 parasites/μL; 95% CI: 2229-3151 parasites/μL) and the lowest in the South-West (geometric mean 1663 parasites/μL; 95% CI: 1296-2133 parasites/μL)(Figure 6). In general, the North had a higher geometric mean asexual parasite density than the South. The 95% CIs for all regions overlapped, however, there was strong evidence for a difference between the asexual parasite density between geopolitical zones (p<0.001). Pairwise comparisons showed that this difference was mainly driven by the North-West and North-Central being significantly different to the Southern geopolitical zones (Appendix 1). Therefore, as a whole the parasite density follows a similar pattern to the parasite prevalence.

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Although there was a trend of higher densities in the Northern zones of the country, there were also variations in the densities seen within states in each zone (Figure 7).

Map

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Figure 7: Geographical heterogeneity in asexual malaria parasite density in children under 5 years in Nigeria in 2018. Geometric mean parasite density was analysed for all 36 states. Names of individual states can be found in Figure 2 and all numbers are provided in Appendix 5.

The results from the geopolitical zone density analysis fit broadly with the previously found malaria prevalence. Therefore, further analysis was conducted to understand if this association between the prevalence and density also occurred in the individual states.The geometric mean asexual parasite density was compared to the asexual parasite prevalence in each state in 2018, finding a moderate positive significant correlation (Spearman’s r=0.39, p=0.02). Therefore, states that had a higher prevalence generally tended to have a higher asexual parasite density (Figure 8).

Diagram

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### Sexual parasite prevalence and density

Overall sexual parasite prevalence was 7.8% (604/7783) and children under 1 year had the lowest prevalence of sexual parasite (4.9%, 95% CI: 3.5-6.7%) and children aged 4 years had the highest prevalence (9.7%, 95% CI: 8.3-11.2%)(Table 3). The sexual prevalence increased with increasing age, except in the oldest age group where it decreased again. The sexual prevalence followed a very similar pattern to the asexual prevalence: it was higher in the northern zones than in the southern (Table 3).

Looking at the association between asexual and sexual prevalence in each state, a strong positive significant correlation was found (Spearman’s r=, p<)(Figure 9). Therefore, states with a higher asexual prevalence tended to have a higher sexual prevalence.

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| --- | --- | --- | --- |
| Table 3: Sexual parasite prevalence according to sex, age and geopolitical zone in 2018 | | | |
|  | **Sexual parasite prevalence, % (n)**  **(N=7783)** | **95% CI (%)** | **p-value\*** |
| **Sex** |  |  |  |
| Male | 8.2 (324/3647) | 7.3-9.1 |  |
| Female | 7.3 (280/3532) | 6.5-8.2 | 0.20 |
| **Age (years)** |  |  |  |
| 0 | 4.9 (37/717) | 3.5-6.7 | <0.001 |
| 1 | 6.3 (103/1534) | 5.2-7.6 |  |
| 2 | 7.6 (124/1514) | 6.3-9.0 |  |
| 3 | 8.8 (154/1598) | 7.5-10.2 |  |
| 4 | 9.7 (160/1493) | 8.3-11.2 |  |
| 5 | 7.4 (26/323) | 4.9-10.7 |  |
| **Geopolitical zone** |  |  |  |
| North-Central | 8.8 (120/1248) | 7.3-10.4 | <0.001 |
| North-East | 7.0 (97/1294) | 5.7-8.4 |  |
| North-West | 12.1 (217/1579) | 10.6-13.7 |  |
| South-East | 4.3 (52/1155) | 3.2-5.6 |  |
| South-South | 4.3 (37/815) | 3.1-5.9 |  |
| South-West | 7.8 (81/1087) | 5.5-8.5 |  |
| 95% CI= 95% confidence interval  \* Pearson’s chi squared test | | |  |

ADD IN GRAPH WITH SEXUAL AND ASEXUAL PREVALENCE AND 95% CIS

Figure 9: Significant positive correlation between asexual and sexual malaria parasite prevalence in each state. 95% CIs are shown.

Sexual parasite densities ranged from 2 to 10,570 parasites/μL, which is a much smaller range than the asexual densities. The geometric mean sexual parasite density was 81 parasites/μL (95%CI: 73-90 parasites/μL), which is significantly lower than the asexual parasite density (p<0.001). There was no significant difference between the geometric mean sexual parasite density between males/females (Table 4).

The geometric mean sexual parasite density was highest in the North-Central and lowest in the South-East (94 and 69 parasites/μL respectively)(Table 4). When looking at the association between sexual parasite density and geopolitical zone there was no evidence of a difference (p=0.63). Similarly, when stratified by age, the geometric mean sexual parasite density was very similar across all age groups (Table 4). The youngest age group did not have a significantly lower density of sexual parasites as compared to the older age groups, which is in contrast to the asexual density findings.

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| --- | --- | --- | --- |
| Table 4: Geometric mean sexual parasite density by sex, age and geopolitical zone in 2018 | | | |
|  | **Geometric mean sexual parasite density (parasites/μL)** | **95% CI (parasites/μL)** | **p-value** |
| **Sex** |  |  |  |
| Male | 85 | 74-98 |  |
| Female | 77 | 66-90 | 0.33\* |
| **Age (years)** |  |  |  |
| 0 | 71 | 47-107 |  |
| 1 | 89 | 69-114 |  |
| 2 | 91 | 70-118 |  |
| 3 | 65 | 54-79 |  |
| 4 | 90 | 74-110 |  |
| 5 | 80 | 45-141 | 0.23\*\* |
| **Geopolitical zone** |  |  |  |
| North-Central | 94 | 75-119 |  |
| North-East | 89 | 66-121 |  |
| North-West | 77 | 65-91 |  |
| South-East | 69 | 48-98 |  |
| South-South | 75 | 48-117 |  |
| South-West | 79 | 60-104 | 0.63\*\* |
| 95% CI= 95% confidence interval  \* Mann-Whitney test  \*\* Kruskal-Wallis test | | |  |

### Plasmodium species

The majority of infections were *P. falciparum*, with an asexual and sexual prevalence of 18.6% and 6.1% respectively (Table 5). Asexual and sexual non-falciparum prevalence in 2018 was 2.9% and 1.7%. When including only infected children, 87.6% (1596/1821) of infections were *P. falciparum*, 6.0% (109/1821) were *P. falciparum/P. malariae* (PF/PM) co-infections, 3.9% (71/1821) were *P. malariae*, 1.3% (24/1821) were *P. falciparum/P. ovale* (PF/PO) co-infections and 1.0% (19/1821) were *P. ovale*.

Co-infections (PF/PM and PF/PO) had the highest geometric mean asexual parasite density with 3021 and 3984 parasites/μL respectively (Table 5, Figure 10a). There was very strong evidence for a difference between the geometric mean asexual parasite density between different *Plasmodium* infections (p<0.001).

In 2018, 29.6% of *P. falciparum* infections contained sexual parasites, whereas this was above 55% for all other *Plasmodium* infections (Table 5). The geometric mean sexual parasite density of *P. falciparum* was 67 parasites/μL (95% CI: 59-75 parasites/μL), which is lower than the other infections (Table 5, Figure 10b).

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| --- | --- | --- | --- | --- | --- | --- | --- |
| Table 5: Plasmodium species distribution in 2018 | | | | | | | |
| ***Plasmodium* species** | **Mean age, years (SD)** | **Sex, % female (n/P)\*** | **Asexual prevalence, % (n/N)** | **Asexual density\*\*, parasites/μL (95% CI)** | **Sexual prevalence, % (n/N)** | **Sexual density\*\*, parasites/μL (95% CI)** | **Sexual stage carriers\*\*, % (n/P)** |
| ***P. falciparum*** | 2.6 (1.4) | 47.4  (756/1596) |  | 2304  (2066-2570) |  | 67  (59-75) |  |
| ***P. malariae*** |  | 46.5  (33/71) |  | 982  (718-1343) |  | 185  (142-242) |  |
| ***P. ovale*** |  | 36.8  (7/19) |  | 622  (322-1202) |  | 173  (71-421) |  |
| **PF/PM** |  | 44.0  (48/109) |  | 3021  (2298-3971) |  | 153  (121-194) |  |
| **PF/PO** |  | 58.3  (14/24) |  | 3984  (2282-6955) |  | 141  (74-270) |  |
| **p-value\*\*\*** |  |  |  | <0.001 |  | <0.001 |  |
| PF/PM = *P. falciparum* and *P. malariae* co-infection, PF/PO = *P. falciparum* and *P. ovale* co-infection, 95% CI = 95% confidence interval.  \* P = total population of individuals positive for that species (includes asexual and sexual stages), some infections only contain sexual stages  \*\* Geometric mean parasite densities shown  \*\* The percentage of positive individuals for that species that contain sexual stages  \*\*\* Kruskal-Wallis test for densities and Pearson’s chi squared test for prevalence | | | | | | | |

# Discussion

Broadening our understanding of malaria endemicity and epidemiology is crucial to combating malaria. Our understanding to date has largely been based on analysis of malaria parasite prevalence. Although a very useful measure for tracking changes in malaria burden, new tools are required to understand how and why changes are occurring. Whilst parasite density has been investigated in earlier studies from Nigeria and other African countries (7,19,32), this study permits a nationwide analysis, allowing the relationship to be understood across geopolitical zones and states. To our knowledge, this is the first study to investigate how changes in parasite prevalence relate to changes in parasite density on a national scale using a randomised sampling process. The main findings from this project are that co-infections consistently contain higher parasite densities than other *Plasmodium* infections and that parasite density is correlated with parasite prevalence. These main findings will be discussed alongside minor themes, such as the age distribution of asexual parasite densities, differences in sexual parasite densities and *Plasmodium* species differences.

For the 2018 data looking at age in relation to densities, when all individuals were included in the analysis compared to when only infected individuals were included in the analysis different patterns emerged (Figure 4). This provided an initial indication that parasite density data could yield additional information on top of prevalence data. When only infected individuals were included in the analysis, there was little difference between the asexual density between age groups (other than the difference seen for children < 1 years)(Figure 4). Hence, age does not seem to have a large effect on asexual densities and is therefore not considered a confounder. This is not overly surprising since we were only investigating a targeted narrow age group (0-5 years). If comparing the result found here with results from community-based surveys previously done in Africa, we see lower levels of parasite density in younger children and the peak comes later when the child is 2-4 years of age (32,36,37). Almost always children under 1 year have lower densities. The peak being at 2 years might suggest that children older than 2 years start to form immunity controlling the malaria infection and resulting in lower asexual parasite densities.

Although not very pronounced, children < 1 years did contain a lower asexual parasite density. One reason for this could be due to maternal antibodies (IgG), which allow some form of protection against high density infections (38). Although the lack of high density infections in young children has been attributed to antibodies acquired from immune mothers, studies have been unsuccessful in finding an association between titres of parasite-specific maternal IgG and parasite density or clinical outcomes (39–41). Another reason for this patterns is that an innate aspect of a young child’s physiology is that they have foetal haemoglobin which is thought to reduce the parasite densities (42). This is believed to occur through a mechanism of reduced parasitised red blood cell (RBC) binding to the vascular cell wall and uninfected erythrocytes. It is thought that this cytoadherence is a contributing factor to the expansion of parasite densities and due to reduced cytoadherence infections occur with lower density. A study found that maternal IgG and foetal haemoglobin work in conjunction with each other to impair cytoadherence (43). On top of these reasons, another explanation is that the youngest children have less exposure to *Plasmodium* parasites due to their lower mobility and physical activity (44). Furthermore, younger children are often wrapped and with their mothers. As children grow, they have a larger surface area and move around more, meaning that exposure may increase possibly leading to higher density infections (45).

Understanding the dynamics of asexual stages is important but tracking changes in the prevalence and density of sexual stages may also be of use, due to their direct relevance in terms of infectiousness and transmission of malaria from humans to mosquitoes (46). Overall findings regarding the sexual prevalence shows that this differs significantly between age, geopolitical zones and states. Sexual parasite prevalence increases with increasing age, which is a different pattern from the sexual parasite density. Overall findings for the sexual parasite density in 2018, showed that they did not differ between age group or geopolitical zones. Sexual parasite prevalence and density is lower than asexual parasite prevalence and density, which is a relationship seen previously (47,48). Reduced densities of sexual stages as compared to asexual stages is thought to occur to evade host immune responses (49). The youngest age group did not have a much lower sexual parasite density than older ages, which is a different pattern to what is seen with asexual parasites. This has been hypothesised to be caused by passive immunity reducing densities of asexual stages but not affecting sexual stage production (32). In looking at how the estimates of parasite density were calculated we can see why microscopy may not be the ideal tool for understanding sexual stage densities. When the final estimate was calculated as 15 parasites/μL, this actually corresponded to only 1 sexual parasite counted (using an example of 520 WBCs counted), which is very low. Studies using molecular techniques have evidenced that the majority of individuals with asexual parasites also carry sexual parasites (46,50), but they are just not detected due to being at submicroscopic levels (51). Through mosquito-feeding experiments it has been shown that having a higher density of sexual stages leads to increased transmission, however, low densities are also able to effectively infect *Anopheles* mosquitoes (52). Furthermore, evidence suggests that when malaria endemicity decreases there is a corresponding increase in sexual stage density and human to mosquito transmission (32,53). Therefore, monitoring and controlling sexual stages are of key importance when malaria endemicity is decreasing.

Three species of *Plasmodium* parasites were observed in this study, with the majority of infections being caused by *P. falciparum* across all three surveys. Results showed that in 2018, nearly 5% of infections were *P. malariae* or *P. ovale* mono-infections, which is a small minority, evidencing the current lower transmission of these species. Additionally, consistently *P. malariae* and *P. ovale* infections contained the lowest density of parasites, which may reflect the prevalence finding in that these infections are likely to be more historic. Due to the lower density of these infections, if sampling occurred with a more sensitive test then we may have picked up more non-falciparum than additional falciparum infections (54). The finding that *P. ovale* and *P. malariae* contain lower densities of asexual parasites is consistent with previous findings (55). Although these infections are considered less severe than *P. falciparum*, there is research to suggest they cause more negative impacts that initially thought. *P. malariae* has been shown to cause nephrotic syndrome, leading to renal failure, can cause reoccurring chronic infections and high anaemia morbidity (56–59). In agreement with this, *P. ovale* has also been associated with severe disease and is known to cause relapses due to dormant hypnozoites (60,61). This is important because in other parts of Africa where *P. falciparum* has been controlled there has been a corresponding increase in other *Plasmodium* species (62,63). This may in part be due to drugs used to treat malaria causing positive selection of non-falciparum species (64). This positive selection may occur based on the fact that it is often only symptomatic cases that are treated, which are more likely to be *P. falciparum*. Additionally, due to the fact that *P. falciparum* has been shown to have a suppressive effect on *P. malariae*, this may also lead to a further increase in this species(65,66). On the other hand, this project shows that PF/PM co-infections contain a high density of parasites and so possibly suppressive effects are not occurring in this population. It is crucial that these parasite species are not be overlooked in future research, especially when control efforts are initiated and evaluated.

Co-infections were seen to consistently contain the highest asexual parasite densities. Co-infection density dynamics could be the result of antagonism, enhancement or additivity between species. Antagonism between species has been reported previously as a result of direct competition for resources or due to cross-reactive immune responses (67,68). However, this data supports the theories of enhancement or additivity, due to the fact that co-infections contain a higher density of asexual parasites. Additivity occurs as a result of multiple species having an additive effect on the density of circulating parasites. A possible but untested theory is that the asexual and sexual stages of *P. falciparum* contain a lower density in mono-infections as compared to in co-infections due to co-infections altering the ability of *P. falciparum* to sequester. Asexual *P. falciparum* parasites are specifically known to adhere to vascular endothelial cells, in a process known as sequestration, and adhere to uninfected RBCs, in a process known as rosetting (69). This results in reduced parasite densities in peripheral blood. Sexual stages of *P. falciparum* are also known to sequester in the bone marrow, meaning they are shielded from splenic clearance, therefore just very immature or late stage sexual stages are present in the bloodstream (70,71). In agreement with these studies, comparison of blood and bone marrow samples in children in Mozambique showed that *P. falciparum* sexual stages were enriched in the bone marrow as compared to the bloodstream (72). This may also provide an explanation as to why we found that *P. falciparum* infections contained a lower percentage of sexual stages as compared to the other *Plasmodium* infections, which would only hold true if *P. falciparum* sexual stages sequestered more than their asexual stages.

Previous work investigating co and mono-infections show differing results. An example of this can be demonstrated through the fact that some studies show that co-infections limit *P. falciparum* sexual stage production (73,74) but in contrast other studies show they enhance production (75,76). It has been suggested that the sequence of the co-infection alters the dynamics, in that sexual stage production is only enhanced if *P. malariae* infection occurs before or at the same time as *P. falciparum* (75). Furthermore, co-infections have been shown to reduce clinical disease in some studies and increase them in others (77–79). Conflicting evidence on the role of co-infections, points to the fact that generalisations should not be made and extrapolating research from one country to another should be limited. A study looking at the density of co and mono-infections in Malawi found that this differed based on the transmission type (perennial/seasonal) and intensity (high/low) in a location (9). Therefore, if transmission intensity continues to change in Nigeria, further differences in the dynamics between co and mono-infections may be seen, which argues for the continued and long-term monitoring of parasite densities. Although this provides evidence for a role for parasite densities to be used to discriminate between infections, this role is not fully understood yet, meaning further research is required. Additionally, there is a far more literature discussing the dynamics of *P. falciparum* and *P. vivax* co-infections, therefore, there is possibly a need to increase research on the co-infections found in this paper, especially as they present at high densities and may possibly cause severe clinical outcomes.

In 2018, the Northern zones tended to have a higher asexual parasite density than the Southern zones, following a similar pattern to the prevalence. A key finding is that asexual density and prevalence data are in agreement at the state level, as shown by the correlation analysis in each year. This low parasite prevalence with a corresponding low parasite density has additionally been found in a cross-sectional survey in the Gambia and Guinea Bissau (80). We have shown that high-endemic areas, where more people are being infected, tend to have higher parasite densities than low-endemic areas. However, there is a range of densities in each setting that can differ by several log orders of magnitude. Numerous hypothesises have been suggested to explain why there is this difference in densities in different transmission settings, with the actual situation likely being a complex interplay between them. Firstly, in low endemic areas, individuals will likely receive fewer infectious bites, meaning that their infections are more likely to be historic. As a malaria infection naturally progresses, parasite densities decrease, meaning that in lower transmission areas a higher proportion of infections will be of low-density (28,31,81). Secondly, areas in Nigeria where there is now low transmission have previously had high malaria transmission (25,26). Consequently individuals in these areas will likely have acquired immunity, so may exhibit enhanced control of parasite densities over and above what would be expected based on the current transmission level (31). This could mean that in the future when completely susceptible individuals, with no historic exposure, are infected then a different density distribution may be seen. Thirdly, in low transmission areas there may be reduced parasite genetic diversity which may facilitate the rapid acquisition of acquired immunity to those specific clones, leading to reduced parasite densities (82,83). In conclusion, in high-endemic areas, individuals are more likely to be superinfected, have a shorter time since infection, get inoculated with multiple species and parasites may have greater genetic diversity. All these factors likely contribute to the higher parasite densities seen in areas with higher prevalence.

The fact that malaria parasite densities are not stable throughout an individual’s infection indicates it may be an unreliable tool to understand changes in endemicity. Individuals in Mali (West Africa) were followed over an extended time period to assess how parasite density fluctuates temporally (84). In excess of 100-fold variations in parasite densities were commonly recorded in individual follow-ups and all positive individuals had at least one negative reading. This provides evidence that peripheral parasite densities in individuals in malaria endemic regions are not static and fluctuations occur regularly. Fluctuations are thought to be the result of the release of sequestered parasite, in conjunction with parasitic intraerythrocytic development synchronisation, immune mechanisms and antigen-switching (84,85). Fluctuations in individual parasite density recordings have additionally been reported in other studies (31,86). Due to this, care should be taken when utilising parasite density measurements as an epidemiological tool for assessing endemicity. This suggests that individual parasite density readings should not be used as a tool to assess endemicity, however, the distribution of parasite densities in a group of individuals has been shown to be of use.

Quantitative estimates of parasite density can add value to discriminating variation among areas of a country in terms of relative infection burden. Parasite density data does differentiate variation on top of that provided by the prevalence among infected children. However, what now needs to be understood is how this data can be used. One issue is whether to use microscopy or molecular methods for detection. Malaria parasite prevalence and density are subject to the sensitivity threshold of detection of the test used (28). There is not a linear relationship between the proportion of positive individuals detected by microscopy and the transmission setting, in that microscopy has reduced sensitivity in low-endemic settings (28,31). Therefore, the usefulness of parasite density as an endemicity index measured by slide microscopy may be limited in countries with low levels of transmission. In a study in West Africa, PCR detected three-times the number of infections as microscopy (80). Although these infections have a lower probability of causing severe disease (87), they have been shown to be an infectious reservoir for onwards transmission through feeding studies (45,46,88). Switching to using molecular methods is not a new concept, having been recommended many times previously (81,85). Molecular methods can be used to look at genome copies, which is an accurate way to measure densities (89). However, one disadvantage is that it cannot easily distinguish between sexual and asexual parasites and additional disadvantages include cost and laboratory differences affecting results. Not only can molecular techniques be used to understand parasite densities but using a proxy measure of density could also be of use, such as a histidine-rich protein 2 (HRP-2) immunoassay, where infections can be understood by looking at proteins that the parasite releases into the bloodstream. This tool may be easier to conduct, less prone to error and can be used as a positive/negative detection but also quantitated (90). Studies have shown the abundance of protein detected is a better measure of absolute number of parasites in a person and HRP-2 quantitative analysis is also more correlated to severe malaria (91,92). Quantitative suspension array technology (qSAT) has been used to detect HPR-2 and lactate dehydrogenase (LDH) (93). qSAT results were shown to be positively correlated with parasite densities determined by qPCR and microscopy, suggesting the test to be a useful tool for predicting parasite densities and being used to assess levels of endemicity.

In each survey density is meaningfully correlated with prevalence, but temporal comparison of parasite density data could be of use to be able to track changes based on control interventions. It has been previously described that asexual and sexual stage densities increase after the cessation of control interventions (32), so looking at changes in parasite density may be able to indicate the successfulness of interventions and indicate where needs renewed control efforts. Further research would be required to understand exactly what changes in density are of importance. A methodological limitation of this study is that we assumed a standard WBC count of 8000/μL in order to estimate the parasite density. Studies in Nigerian children have shown that this approximation may lead to an over-estimation of the parasite density (94). On the other hand, this approximation method has been shown to have a very similar distribution to when absolute WBC counts are used (95). Additionally, this limitation has less relevance to this study due to its comparative nature and as long as nationally the same protocol is utilised.

Future recommendations are that all Demographic and Health Surveys should include density data. However, if this was to be implemented in the future, issues of standardisation across years will be important. The data needs to be comparable and for this to occur there needs to be standardisation of protocols. Temporal comparisons of the data could occur if each survey was being conducted in the same laboratory using the same protocol. However, training and expertise in slide processing, microscopy and data entry may change over time leading to differential results (96). Another methodological issue that could affect the results are batch effects, where drying, staining or humidity affect the outcome (97). However, by designing and conducting the surveys knowing they will be compared, this would help reduce systematic biases and errors, therefore providing more robust data. Furthermore, malaria is highly seasonal in Nigeria, in that it regularly and predictably changes annually based on climatic conditions, meaning that results will differ based on the timing of the surveys (98). Seasonality has marked effects on the parasite prevalence and density, generally causing an increase in both during the wet season (8,64). Climatic conditions affect *Anopheles* egg development, rates of sporogony, mosquito longevity and feeding frequency (99–101). In a previous study in Burkina Faso, a positive association between rainfall and *P. falciparum* density was observed but this was not observed with *P. malariae* or *P. ovale* (64). Therefore, climatic conditions before a survey may lead to different species density distributions. This shows that the surveys should be conducted in exactly the same time frame in the year and possibly climactic conditions should be measured due to year-on-year variations. If data on climatic conditions was collected, then during analysis it could be controlled for.

This paper asserts that parasite density could be a useful epidemiological parameter to assess malaria endemicity. Severe clinical outcomes are known to be associated with high parasite densities of *P. falciparum* and the density of sexual stages have been shown to relate to transmission of parasite to mosquitoes (15,102,75). Therefore, in the future when looking at which control interventions are successful, parasite density data can be of use. To reignite progress towards successful malaria control, it is crucial that a combination of control interventions are utilised and these need to be targeted and optimised. Targeted interventions could occur as a result of monitoring parasite density, possibly through a combination of microscopy, molecular techniques or HRP-2/LDH as a proxy, bringing forward the increasingly popular idea of surveillance as a public health intervention.