In-trap DNA contamination:
tsetse xenomonitoring methods can lead to biased estimates of *Trypanosoma brucei* infection

LIVERPOOL SCHOOL OF TROPICAL MEDICINE
Since 1898

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1. BACKGROUND AND OBJECTIVES

Tsetse flies (*Glossina sp.*) are vectors of subspecies of *Trypanosoma brucei* which cause human African trypanosomiasis (HAT). Catching tsetse and screening them for the presence of *T. brucei* is an important method of **HAT surveillance.** Classically, individual tsetse were dissected and subjected to microscopic analysis to identify trypanosomes if present. However in the 'genomics age' such techniques are being replaced by **molecular xenomonitoring**[1,2] (Fig. 1). Assays using a range of *T. brucei* genomic targets have been developed for this purpose, including the 10,000-copy *T. brucei* tandem repeat (TBR) region[3,4]. The use of **such highly sensitive targets in an end-point assay such as PCR can lead to difficulties in differentiating true biological infection and are vulnerable to DNA cross-contamination**. Such contamination may occur at capture, when live tsetse are held in a small trap cage. Several xenomonitoring studies that have used TBR-PCR have reported higher-than-expected proportions (5% or more) of *T. brucei*-positive flies[5–8]. **Therefore, this study set out to investigate whether it is possible for *T. brucei* infected tsetse to contaminate uninfected tsetse with *T. brucei* DNA when housed in the same trap.**

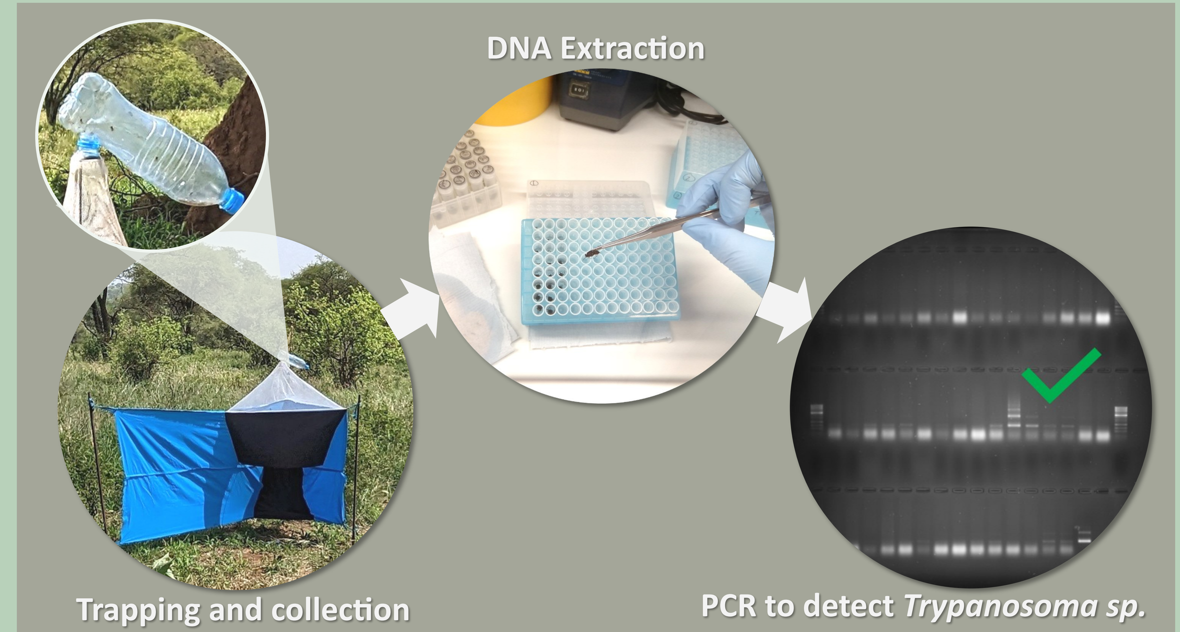

Figure 1: A diagram depicting the typical tsetse xenomonitoring workflow

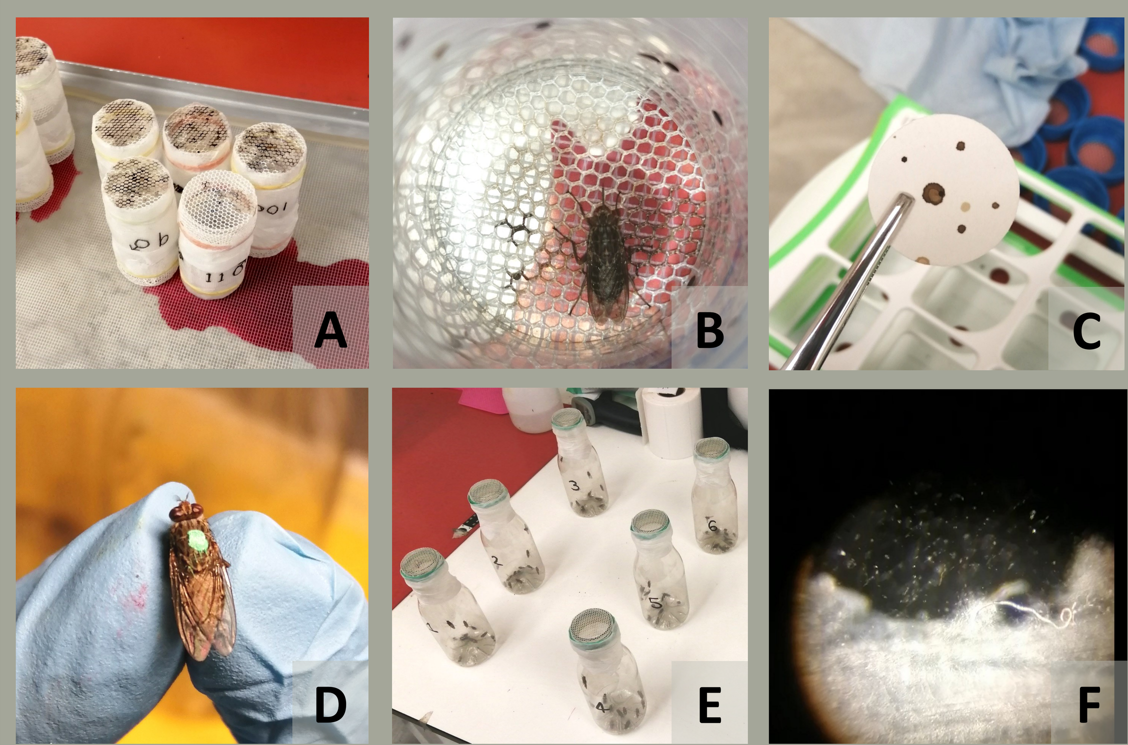

Figure 3: (A) and (B) show tsetse being blood-fed in solitary cells; (C) collection of tsetse faecal samples on filter paper; (D) an infected fly marked with green oil paint; (E) experiment trap cages; (F) dissected tsetse mid-gut tissue infected with *T. brucei*.

2. METHODS

As indicated in Fig. 2, a total of 140 teneral *G. morsitans morsitans* were **fed a bloodmeal spiked with *T. brucei brucei*** TSW 196 and maintained in solitary cells (Fig.3A-B). TBR-qPCR screening of tsetse faecal samples (Fig.3C) collected 9-14 days post inoculation (dpi) were used to determine individual fly infection status. At 19dpi, 48 **infected flies (IFs)** were selected and marked with a unique label (Fig.3D). IFs and 96 (not inoculated) **uninfected flies (UFs)** were placed in plastic bottles similar to the cages used with traps (Fig.3E). The numbers of IFs and UFs in the bottles was varied according to four classes of treatment (Fig.2). **The four treatments comprised IF:UF in the ratios**: **(T1) 9:3**, **(T2) 6:6**, **(T3) 1:11** and **(T4) 0:12**. Each treatment was replicated three times (A, B and C). After 24-hour incubation, all flies that had ingested an infectious bloodmeal (n=110) were dissected and microscopically analysed to determine infection status (Fig.3F). All tsetse samples (n=206) then underwent DNA extraction and **screening using TBR-PCR, TBR-qPCR and GPI-PLC-qPCR.**

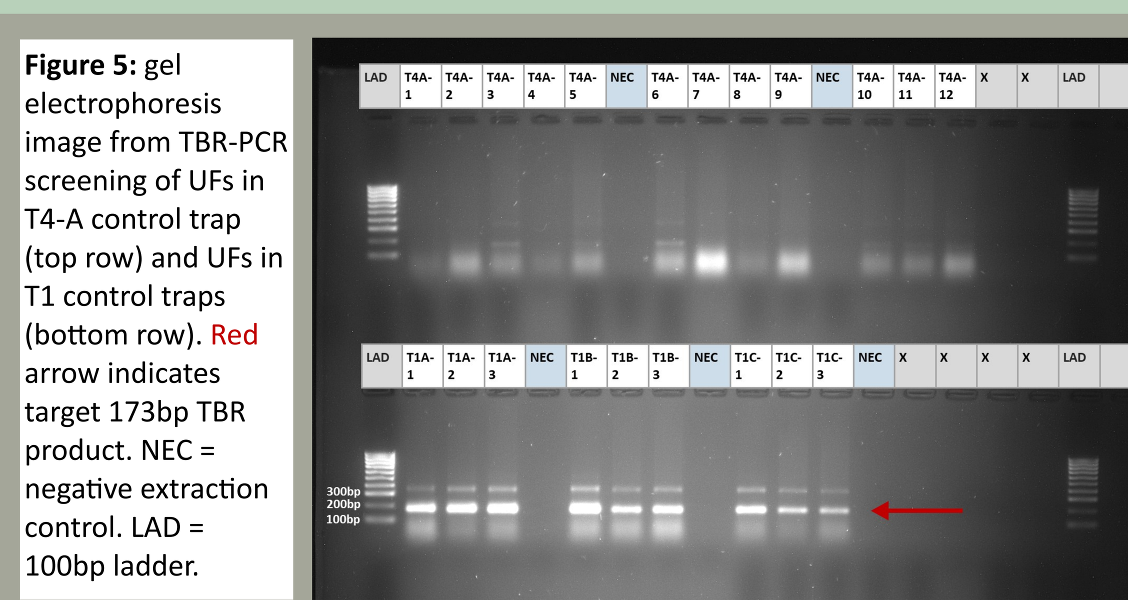

Figure 5: gel electrophoresis image from TBR-PCR screening of UFs in T4-A control trap (top row) and UFs in T1 control traps (bottom row). Red arrow indicates target 173bp TBR product. NEC = negative extraction control. LAD = 100bp ladder.

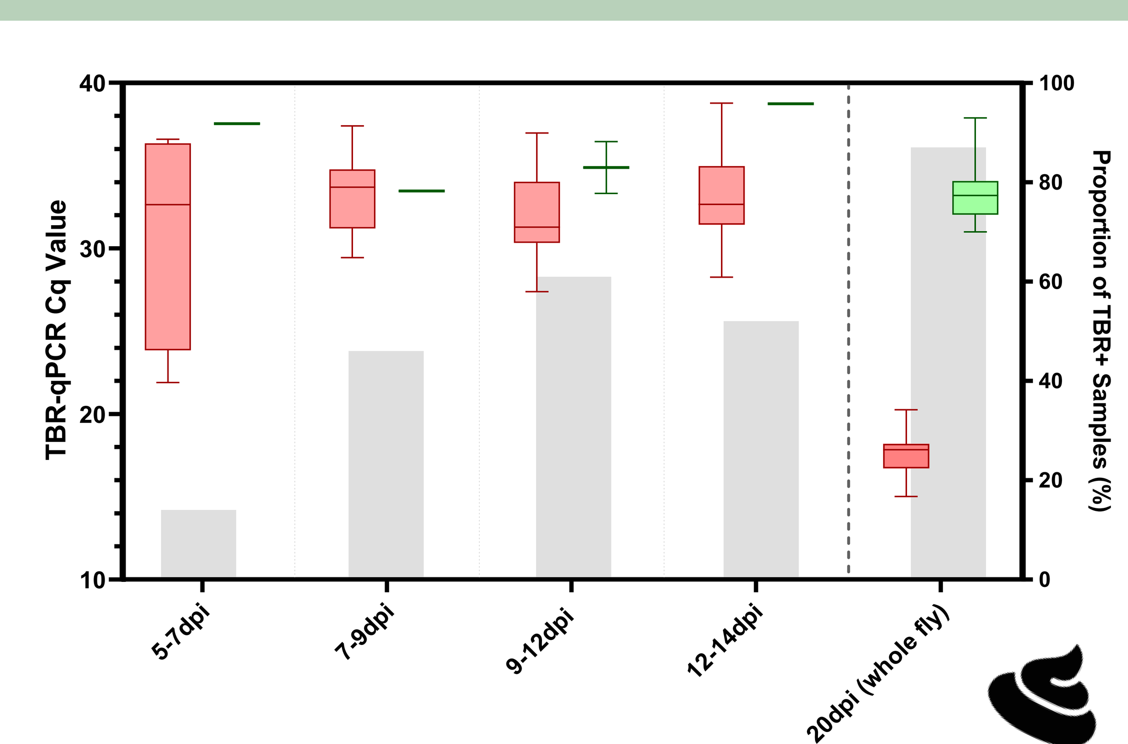

Figure 6: A box-and-whisker plot (left axis) showing Cq values obtained from **TBR-qPCR screening of faecal samples** at four timepoints and eventual whole fly DNA. Samples from infected flies are in red, samples from uninfected flies are in green. The bars (right axis) shows the proportion of faecal samples recording TBR-qPCR amplification (where samples were available).

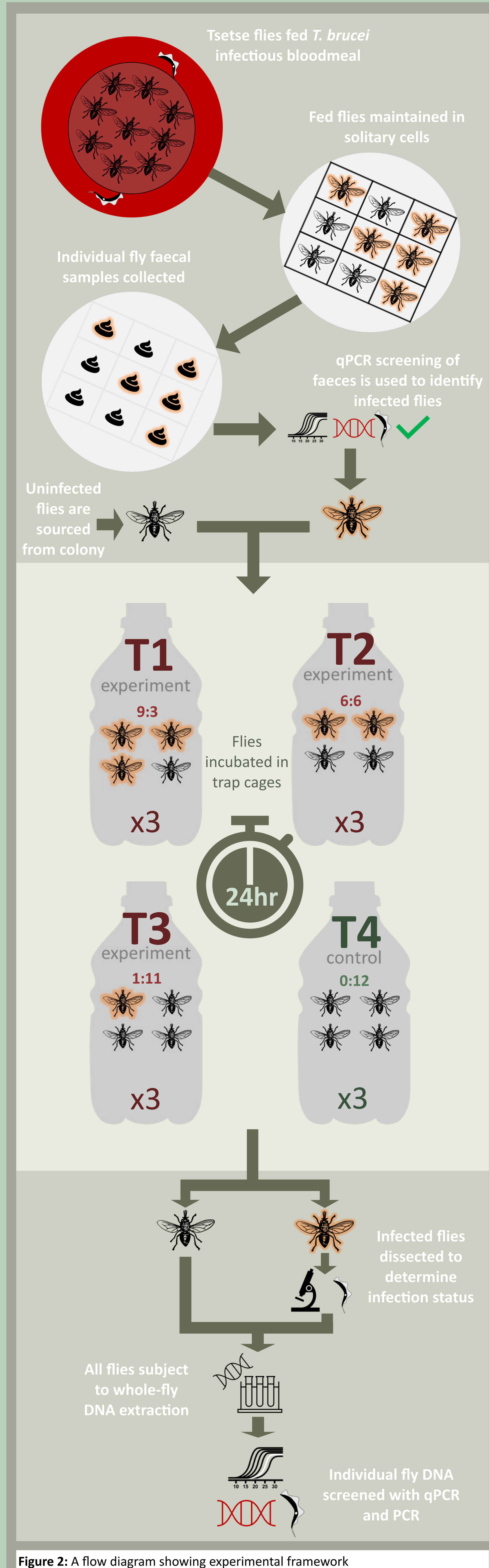

Figure 2: A flow diagram showing experimental framework

3. RESULTS

Infected flies can contaminate uninfected flies. Screening of 48 IFs by TBR-qPCR revealed Cq values between 14.46-21.57 (mean=17.74, SD=0.7458) in 100% of flies. However UFs also contained TBR target DNA, the quantity of which **varied according to the proportion of infected flies within the trap** (Fig. 4A). For T1 and T2, 100% of UFs had detectable DNA with mean Cqs of 26.72 (±0.7498 SD) and 29.84 (±1.375 SD) respectively (Fig.4A). For T3, 91% UFs had detectable DNA with a mean Cq of 33.70 (±1.352 SD)(Fig.4A).

Contamination evident even with lower-sensitivity single-copy target assay. Screening of UFs by GPI-PLC-qPCR revealed 100% of T1 UFs recorded amplification (Cq mean=33.10 ±1.387 SD), 72.2% of T2 UFs (Cq mean=32.57 ± 1.324 SD), 9.1% of T3 UFs (Cq mean=34.69 ±2.524 SD) and no amplification across T4 UF controls (Fig.4B).

Contamination detected in control traps placed close to experiment traps, but not in control trap placed in separate room. Low-level amplification was detected in 50% of UFs in T4-A and T4-B (placed within close proximity to infected flies) of mean Cq 35.23 ±1.396 SD. UFs in T4-C (placed in a separate room) recorded no amplification.

Faecal screening can determine tsetse midgut infection (Fig.6). Microscopy revealed that 100% (n=48) of IFs selected for experiments had developed mature midgut infection. **Faecal samples collected 9-14dpi that tested positive (TBR-qPCR) were highly likely to be from an infected fly,** with diagnostic **PPV of 91% and NPV 85%.**

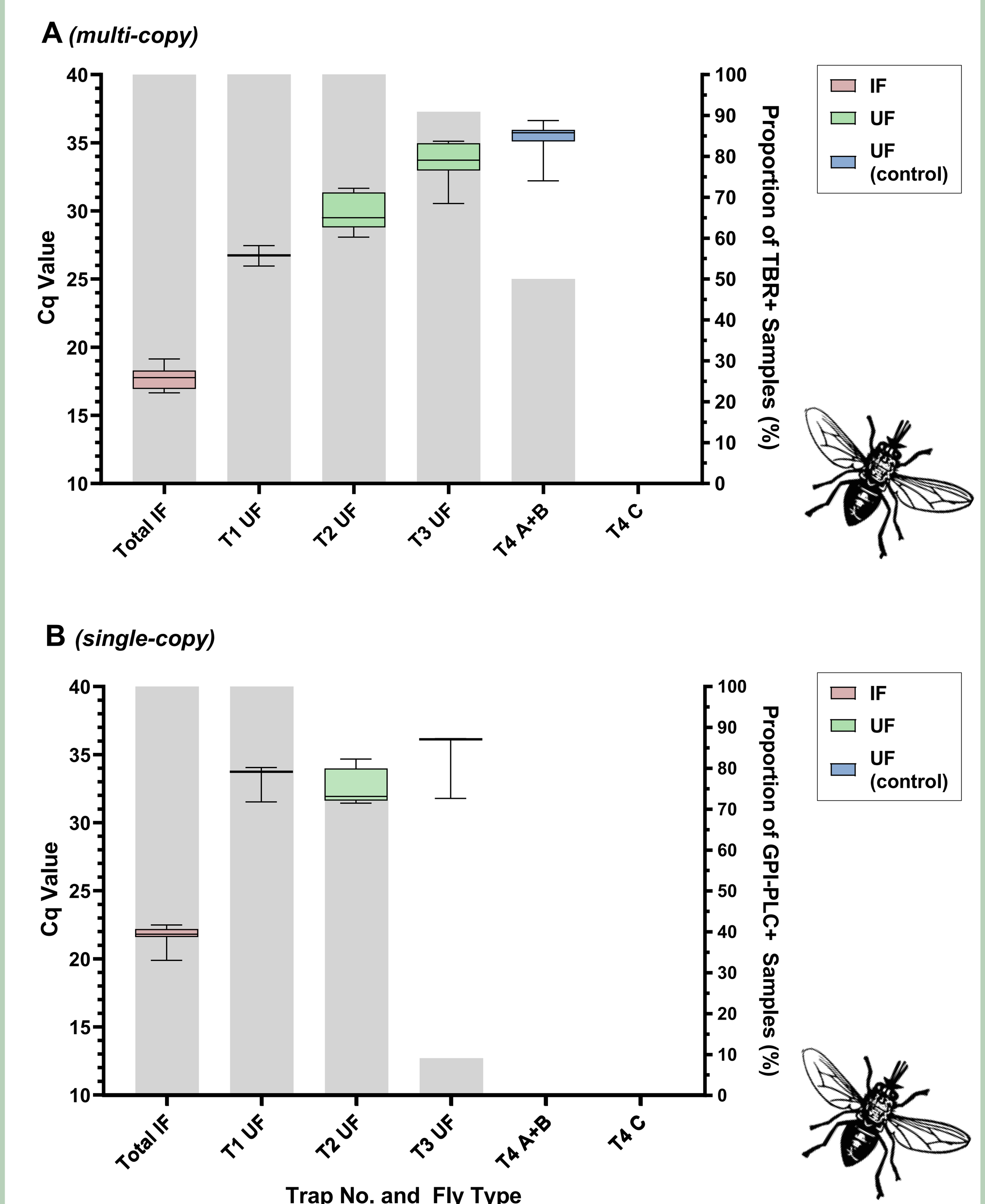

Figure 4: box-and-whisker plots showing Cq value data from *T. brucei* **(A)** multi-copy target TBR-qPCR screening and **(B)** single-copy target GPI-PLC-qPCR screening of infected flies (IF) and uninfected flies (UF) across four trap types (T1-T4). Grey bars display proportion of samples recording amplification using respective qPCR assays.

4. CONCLUSIONS

- **Infected tsetse can contaminate uninfected flies with *T. brucei* DNA within a trap cage** and the level of this contamination can be extensive.
- Whilst simple PCR may overestimate infection rates, **quantitative PCR offers a means of identifying 'true' infections.**
- We have also described **a novel tsetse faecal screening method to determine *T. brucei* midgut infection status ante-mortem**. It is sensitive and more efficient than classic 'probing' method.
- Further study required in the field and with different trap cage types.

References: [1]Cunningham, L. J. *et al.* Illuminating the Prevalence of Trypanosoma brucei s.l. in Glossina Using LAMP as a Tool for Xenomonitoring. *PLoS Negl. Trop. Dis.* **10**, e0004441 (2016). [2]Garrod, G. *et al.* A pilot study demonstrating the identification of Trypanosoma brucei gambiense and T. b. rhodesiense in vectors using a multiplexed high-resolution melt qPCR. *PLoS Negl. Trop. Dis.* **14**, e0008308 (2020). [3]Ahmed, H. A., Picozzi, K., Welburn, S. C. & Macleod, E. T. A comparative evaluation of PCR- Based methods for species- Specific determination of African animal trypanosomes in Ugandan cattle. *Parasites and Vectors* **6**, (2013). [4]Moser, D. *et al.* Detection of Trypanosoma congolense and Trypanosoma brucei subspecies by DNA amplification using the polymerase chain reaction. *Parasitology* **99 Pt 1**, 57–66 (1989). [5]Karshima, S. N., Ajogi, I. & Mohammed, G. Eco-epidemiology of porcine trypanosomosis in Karim Lamido, Nigeria: Prevalence, seasonal distribution, tsetse density and infection rates. *Parasites and Vectors* **9**, 1–9 (2016). [6]Ochi Yamba-yamba, E. B. *et al.* Performance assessment of molecular and microscopy tests for detection of trypanosoma species in glossina fuscipes fuscipes (diptera: glossinidae) midguts in Kajo-Keji county, South Sudan. *Int. J. Bioassays* **4**, 3910–3914 (2015). [7]McNamara, J. J., Laveissière, C. & Masiga, D. K. Multiple trypanosome infections in wild tsetse in Côte d'Ivoire detected by PCR analysis and DNA probes. *Acta Trop.* **59**, 85–92 (1995). [8]Musaya, J. *et al.* Polymerase chain reaction identification of *Trypanosoma brucei rhodesiense* in wild tsetse flies from Nkhotakota Wildlife Reserve, Malawi. *Malawi Med. J.* **29**, 11–15 (2017).

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