# *Anopheles* mosquitoes exposed to long-acting antimalarials via drug-spiked bloodmeal absorb drug but do not suffer fitness costs

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# Abstract

The World Health Organization's recommendations regarding the use of antimalarials for the prevention of malaria in endemic areas have greatly expanded, allowing more flexibility in the demographic groups and regions where chemoprevention and mass treatment are acceptable. An overlooked aspect of expanding human population-level drug exposure is the downstream impact of ingested drug on the mosquito vector. Data suggest both infected and uninfected *Anopheles* mosquitoes re-feed often with ≥4 blood meals during their lifespan. This provides repeated opportunities for mosquitoes to ingest drug via bloodmeals taken from people with antimalarials in the bloodstream and raises questions as to whether exposure may impact the mosquito itself, and/or parasites developing within the infected mosquito. We investigated the impact of exposure to physiologic levels of commonly used long-acting human antimalarials in the *Anopheles* mosquito via drug-spiked blood feeds. We did not observe any significant differences in mosquito feeding, behavior, fertility, or viability after ingestion of amodiaquine, desethylamodiaquine, piperaquine, and sulfadoxine-pyrimethamine in either lab-reared *An. gambiae* or field-derived *An. coluzzii* mosquitoes. Interrogating drug distribution within mosquitoes utilizing LC-MS/MS, desethylamodiaquine, the longer-acting active metabolite of amodiaquine, was fed at 2 concentrations (1/2X and 2X $C_{max}$) with drug subsequently detected in a dose-dependent manner in pooled whole mosquitoes, midguts and hemolymph. This was significant for whole mosquitoes harvested at 24hrs and 120hrs, and midguts harvested at 24hrs. Between 24 to 120 hrs, drug decreased in midguts but increased in hemolymph. Our results show biochemical evidence of antimalarial absorption into *Anopheles* hemolymph following bloodmeal

ingestion. These studies lay the foundation for future work to assess the impact of vector-stage antimalarial drug exposure on parasite progression throughout development in the mosquito, which could in turn have important implications for transmission dynamics and drug resistance spread.

## Author summary

Drug resistance to first-line antimalarials has emerged in multiple African countries. A better understanding of antimalarial drug resistance emergence and spread is critical in preventing further morbidity and mortality. Millions regularly receive antimalarials for prophylaxis and mass treatment that are purposefully long-acting. *Anopheles* mosquitoes re-feed frequently, and these malaria vectors (both infected and uninfected) routinely feed on people whose blood contains these long-acting antimalarials. Parasites take approximately 10 days to develop within the mosquito. There is published precedent that several antimalarials can act upon vector-stage parasites, yet any potential impact of antimalarials on mosquitoes and/or parasites developing within has been largely overlooked. We questioned whether antimalarials ingested in mosquito bloodmeals could influence parasite development and drug resistance selection. As initial investigations, we exposed uninfected *Anopheles* mosquitoes to commonly used long-acting antimalarials via drug-spiked bloodmeals, mimicking predicted physiologic drug exposure. Investigated drugs did not impact mosquito viability. However, mass spectrometry confirmed drug absorption in whole mosquitoes, as well as within midguts and circulatory fluid, several days after feeding, demonstrating that mosquitoes can ingest key drugs without suffering fitness costs, and these drugs can persist in

mosquitoes. This highlights the potential for antimalarials to impact parasite development and drug resistance selection within the mosquito.

# Introduction

Despite gains in detection, treatment, and prevention strategies over previous decades, including recent approval of the first malaria vaccines, malaria remains a human pathogen of significant consequence, causing 597,000 deaths and 263 million cases in 2023 [1]. Unfortunately, formerly decreasing morbidity and mortality trends have now flatlined – particularly worrisome as insecticide resistance in the *Anopheles* mosquito vector and drug resistance in the predominant *Plasmodium falciparum* parasite species rapidly emerges and spreads [2–5]. Malaria control efforts are necessarily multipronged and include the widespread use of long-acting antimalarials for both chemoprevention and treatment. In the West African Sahel region, seasonal malaria chemoprevention (SMC) has been deployed to children under 5 years old as prevention during high transmission periods since ~2014. Thus far, administration of the long-acting drugs sulfadoxine (SDX)-pyrimethamine (PYR) (referred to as SP together) plus amodiaquine (AQ) (SP-AQ combined for SMC) has been the sole SMC regimen, and was administered to 53 million children per monthly cycle in 2023 [1,6–8] in countries with seasonal malaria transmission. SP-AQ for SMC is now also being considered for use in older children. Other WHO-endorsed long-acting prophylaxis and disease reduction strategies include intermittent preventative treatment in pregnancy (IPTp; all pregnant women in malaria endemic areas), which also employs SP (though dihydroartemisinin (DHA) + piperaquine (PPQ) (DP in combination) is also being considered), and mass

96 drug administration (MDA; community wide treatment in moderate-high transmission
97 areas to reduce disease burden), which typically involves DP. First-line antimalarial
98 treatment typically combines a short-acting artemisinin derivative with a long-acting
99 partner (e.g. artesunate-AQ (ASAQ), artemether-lumefantrine (AL), or DP [6].
100
101 Recommendations for community antimalarial dosing in the context of prophylaxis and
102 mass treatment are now expanding, which is a “paradigm shift” according to the WHO
103 because its guidelines no longer specify strict age groups, transmission intensity
104 thresholds, numbers of doses or cycles, or specific drugs [6]. Given the intentional long-
105 acting nature of many antimalarials, with expanded use, it is anticipated that large
106 proportions of a population could have drugs in their circulatory system for a majority of
107 the transmission season(s). In fact, there is already abundant evidence of widespread
108 circulating levels of human antimalarials in malaria endemic areas. Our group
109 demonstrated that 90% of Ugandan children treated with DP had PPQ detectable in
110 plasma at their next malaria episode, even those occurring up to 126 days post-
111 treatment [9]. A Tanzanian study found that 74% of people had ≥1 antimalarial
112 detectable despite no reported intake for 28 days prior [10]. The impact of such high
113 levels of extended drug presence in human populations raises inevitable questions
114 regarding how such exposure could facilitate drug resistance emergence and/or spread.
115
116 Malaria drug resistance is generally thought to arise through mutation and selection
117 during symptomatic human blood stage infection, when both parasite replication and
118 drug pressure are greatest [11]. In contrast, mass drug intervention is thought to reduce

symptomatic cases and transmission, and in turn, pose a lower risk of resistance emergence and selection [12,13]. However, very little is known about how drugs ingested by mosquitoes during bloodmeals could impact susceptible parasite forms in the mosquitoes, as well as parasite resistance selection within the vector [14].

Revisiting the nuances of vector feeding habits and parasite development within the vector (known as sporogony) is central to this question. *Anopheles spp.* mosquitoes become infected after feeding on a person harboring gametocytes (the *Plasmodium* sexual stage); in the vector midgut parasites undergo sexual replication, with subsequent oocysts beginning to form within ~24 hours. Oocysts then further develop under the mosquito midgut epithelium for ~9-14 days before releasing thousands of sporozoites which migrate via hemolymph (circulatory fluid) to salivary glands for entry into a new host upon next feed [15–17]. However, mosquitoes do not only feed twice during their lifetime [18–21]. The *Anopheles* vector can survive up to ~20 days and have multiple egg laying cycles in the wild. They re-feed during each gonadotrophic cycle and sometimes within a single gonadotrophic cycle (particularly given nutrient demands and feeding disruptions) [18,21,22] – thus it would be common to have ≥4 blood meals per lifetime [23]. Molecular analyses of bloodmeal DNA confirm multiple host feeds on capture [21,22,24,25]. In fact, re-feeding in the lab accelerates oocyst growth [18,19] and sporozoite development time, thereby shortening the incubation period and potentially increasing transmission risk [18].

With population-level drug deployment, infected mosquitoes would frequently be

142 exposed to antimalarial-containing bloodmeals. Evidence from others supports that
143 mosquitoes can absorb drugs during feeding. Duthaler, et al. demonstrated that the
144 endectocide ivermectin can be absorbed in *Aedes* mosquitoes [26]. However, the
145 question as to whether exposure to antimalarials within bloodmeals could significantly
146 impact parasite development during sporogony has largely been unexplored. A seminal
147 study on antimalarial activity across parasite life cycle stages supported potential anti-
148 oocyst activity of several current antimalarials [27]. Although these data were indirectly
149 inferred via eventual oocyst counts following drug exposure in gametocyte cultures,
150 results support the theoretical potential for drugs to act during sporogonic stages. The
151 authors reported that AQ, sulfamethoxazole, PYR, lumefantrine, mefloquine,
152 pyronaridine, and an 8-aminoquinoline analog NPC1161B have potential activity against
153 either gametocyte/exflagellation, ookinete, or oocyst stages [27]. Others have also
154 reported incorporating drug exposure into infectious feeds can impact resultant
155 mosquito oocysts and sporozoites [28–31], although much of this work utilized
156 antibiotics or antimalarials not commonly used for mass prevention and treatment.
157 Specific attention to antimalarials in widespread use and deployed with the goal of
158 maximizing population coverage has not been examined.
159
160 To address this, we first investigated whether bloodmeal exposure to AQ, SP, and PPQ
161 have direct impacts upon the *Anopheles* malaria vector at levels expected to be present
162 in human blood. If so, such drug exposure would complicate further exploration of the
163 effect of ingested antimalarials on parasite development within the mosquito, since the
164 parasites require mosquitoes to survive for at least 9 days to complete sporogony and

be transmitted onward. We focused on AQ/desethylamodiaquine (DEAQ), SP, and PPQ because of their relevance in prophylaxis and treatment, long half-lives, and literature supporting possible vector-stage activity [27]. We next examined whether these key chemoprophylactic antimalarials could be absorbed beyond the mosquito midgut, as such data would support the potential for them to act on parasites developing within the mosquito.

# Results

To investigate effects of mosquito ingestion of commonly used long-acting human antimalarials, we spiked drug into mosquito bloodmeals. We focused on AQ and DEAQ, the longer-acting metabolite of AQ that has potent anti-parasitic activity [32,33], as well as PPQ and SP. We used ivermectin (IVM) as a control due to its endectocidal properties [34] and related trials of mass distribution for malaria control [35–39]. The PK of these drugs are well characterized in humans by our group and others [33,40–46], informing relevant exposure concentrations in experiments performed here.

## Ingestion of drug solvents did not impact vector fitness of *Anopheles* mosquitoes.

We first examined whether the solvents used to dissolve drug compounds had any impact on mosquito fitness at concentrations used with subsequent planned drug-spiked blood feeds. Solvents included acetonitrile (ACN):$H_2O$ 1:9 v/v with 0.5% formic acid (for AQ, DEAQ, PPQ), ACN:$H_2O$ 1:1 v/v with 0.1% hydrochloric acid (SDX),

ACN:H2O 1:1 v/v/ with 0.5% formic acid (PYR), and DMSO (IVM). We calculated a maximum possible solvent exposure to involve a 1:833 plasma dilution, based on a modest drug dissolving target of 1 mg/ml, a standard example $C_{max}$ of 300 ng/ml, plans to use drug concentrations up to twice the reported $C_{max}$, and conservative estimates of pipetting variability. Feeding our lab colony *Anopheles gambiae* mosquitoes solvent diluted into plasma at 1:833 combined with packed RBCs to 40% hematocrit did not affect mosquito feeding behavior, survival, or fertility globally (S1 Fig and S1-S5 Tables). Survival curves were significantly different when comparing a few conditions (ACN 1:1 + 0.1% HCl lower survival than ACN 1:1 (p=0.01) and ACN 1:1 + FA 0.5% (p=0.0046); and HCl 0.1% alone lower survival than ACN 1:1 (p=0.0032), ACN 1:1 + FA 0.5% (p=0.0014), and FA 0.5% (p=0.0381)) but no solvent condition trialed was significantly different from the un-spiked blood only control (S4 Table). Subsequent drug stocks were made at 10 mg/ml for each compound, further reducing solvent concentration in drug-spiked bloodmeals. Given these results, DMSO (solvent for IVM) was not tested as a separate solvent control condition in subsequent experiments for lab mosquitoes.

## Ingestion of antimalarials AQ, DEAQ, PPQ, and SP has no significant impact on multiple measures of long-term lab-adapted *Anopheles gambiae* mosquito fitness

The malaria parasite's ability to complete sporogony is significantly impacted by mosquito behavior and meal seeking, nutrient expenditure on ovulation, and overall vector lifespan [47]. To assess these effects, we next investigated the impact of drug

209 ingestion on *Anopheles gambiae* mosquitoes from our laboratory colony. Drugs stocks
210 of 10 mg/ml were diluted into plasma and combined with packed RBCs at 40%
211 hematocrit for mosquito feeds. Target drug concentrations were selected based on
212 literature reported $C_{max}$ values following standard dosing ($C_{max}$ approximated as 20
213 ng/ml for AQ [42], 500 ng/ml for DEAQ [42], 300 ng/ml for PPQ [41,43,44], 100 µg/ml for
214 SDX [40,45], 400 ng/ml for PYR [40,45], 50 ng/ml for IVM [46]). Each drug was tested at
215 concentrations ranging from one-half to twice $C_{max}$. For SDX and PYR, given these
216 components are dosed together, the SP combination was also tested. Negative controls
217 included un-spiked blood and blood spiked with the relevant solvent at the equivalent
218 volume and concentration. In addition, IVM served as a positive control given expected
219 endectocidal activity upon ingestion.
220
221 Spiking drug into bloodmeals did not produce significant differences in mosquito feeding
222 behavior when comparing control un-spiked blood feeds to each drug-spiked bloodmeal
223 condition, as measured by percentage of female mosquitoes offered bloodmeal that fed
224 (blood visible in abdomen) (Fig 1 A-C and S1 Table). Using an alternative chi-squared
225 based analysis of fed versus unfed mosquitoes under each condition yielded some
226 statistical differences. However, these differences were not consistent between drug
227 concentrations nor even within the same control conditions (e.g. IVM across
228 experimental groups) (S1 Supporting text and S2 Table).
229
230 **Fig 1. Tested antimalarials do not significantly impact lab-adapted *An. gambiae***
231 **behavior, viability, or fertility.**

232 Human plasma was spiked with antimalarials AQ, DEAQ, PPQ, or SP at levels ranging
233 from one half (1/2X) to twice (2X) the average reported $C_{max}$ of standard human dosing,
234 or with the endectocide IVM (at 1X $C_{max}$), and combined with RBCs at 40% hematocrit
235 for mosquito feeds. Negative controls included un-spiked blood ("Blood only") or blood
236 spiked with drug solvent alone ("Solvent"). Solvents were: ACN:H2O 1:9 + 0.5% FA for
237 AQ, DEAQ, PPQ; ACN 1:1 H2O + 0.1% HCl for SDX; ACN 1:1 H2O + 0.5% FA for PYR
238 (for SP experiments, "Solvent" represents the combination of both SDX and PYR
239 solvents). Approximately 50 mosquitoes were fed per group. A-C) Mosquito feeding
240 behavior plotted as percent taking a bloodmeal. D-F) Mosquito survival post drug
241 ingestion plotted in a Kaplan-Meier survival curve (monitored for 14 days). G-I)
242 Mosquito fertility assessed by total eggs laid per condition (n=5 mosquitoes monitored
243 per experiment). Each graph represents combined data from 3 independent
244 experiments (with 3 data points per condition/time, except for a few data points for
245 survival monitoring resulting in fewer than triplicate observations for AQ and DEAQ Day
246 12-14, SP Day 9, 10, and PPQ D9 (2 data points each); and PPQ D 11, 13 (1 data point
247 each)). In A-C and G-I, means are plotted with error bars representing SD.
248
249 Fed mosquitoes were monitored up to 14 days, towards the longer end of average
250 expected duration of *P. falciparum* sporogony [17]. In activity monitoring experiments,
251 consistent differences in behavior were not observed for any experimental condition
252 other than following IVM feeds, which reliably led to loss of mobility notable within ~24
253 hours (data not graphed). While survival rates naturally vary by individual experimental
254 blood feed, particularly depending on age of mosquitoes at time of feed (ranging from 5-

10 days), we also did not observe consistent negative impacts of drug ingestion on mosquito survival compared to control un-spiked or solvent-spiked bloodmeals other than expected effects from IVM (Fig 1 D-F, S1 Supporting text, and S3 Table). (Amongst multiple comparison analyses of all conditions, there were very limited conditions where survival curves were significantly different (S1 Supporting text, S4 Table).) Finally, there were also no significant differences in fertility measures following ingestion of any of the compounds compared to unaltered blood as indicated both by total egg counts per mosquitoes monitored (Fig 1 G-I; S5-S6 Tables) and egg counts per individual mosquitoes or percent of mosquitoes laying eggs (S2 Fig; S5-S6 Tables).

In contrast, IVM control feeds were consistently lethal, confirming successful target drug dilution precision and relevance of this experimental drug-spiked blood feeding system, Notably, while feeding behavior was unaltered in the presence of IVM, within 24 hours, the majority of mosquitoes lost mobility with significant numbers dying by 48 hours (see below and S3 Table). Because of this rapid alteration in behavior, IVM-fed mosquitoes were not subjected to individual fertility monitoring (otherwise drowning in forced egg laying conditions). When IVM-fed mosquitoes were allowed access to a communal egg laying water cup, they did not lay eggs (though dissections indicated eggs had formed, suggesting the reason for fertility absence was likely due to the neurologic and behavioral impacts of IVM). This is consistent with the known mechanism of action of IVM being a neurotoxin that binds to invertebrate-specific glutamate-gated chloride channels, causing hyperpolarization and paralysis [48].

## Ingestion of long-acting antimalarials did not significantly impact mosquito fitness of *Anopheles coluzzii* mosquitoes in Burkina Faso

Over many generations of lab adaptation, mosquito behavior and metabolism could be altered in ways that affect the impact of drug ingestion on fitness. Thus, we next investigated antimalarial feeding in field-derived mosquitoes from a routinely outbred *Anopheles coluzzii* colony in Burkina Faso. The same experimental design was employed, although the blood source differed, dissolving drug into commercial AB serum before mixing with donor packed RBCs from a community blood bank at 40% hematocrit. Field mosquito feeding rates using membrane feeders and stored blood components were significantly lower than for lab-adapted *Anopheles gambiae* tested at the Yale insectary (mean 51.6% of field mosquitoes feeding versus 84.1% of lab mosquitoes across all feeding experiments and conditions, $p<0.0001$), even with pre-feeding starvation for 24hrs. However, feeding rates did not differ by condition (percentage of female mosquitoes offered bloodmeal that fed, with blood visible in abdomen (Fig 2 A-C; S1 Table)). As above, using an alternative chi-squared based analysis of fed versus unfed mosquitoes under each condition yielded some statistical differences comparing mosquitoes fed spiked bloodmeals to those fed an unaltered bloodmeal or relevant solvent-spiked bloodmeal (S1 Supporting text, S2 Table). However, similar to the *An. gambiae* feeds, these differences were not consistent between drug concentrations nor even within the same control conditions across different feeding groups.

300

301 **Fig 2. Tested antimalarials do not significantly impact field *An. coluzzii* behavior**
302 **or viability.**

303 Human serum was spiked with antimalarials AQ, DEAQ, PPQ, or SP at levels ranging
304 from one half (1/2X) to twice (2X) the average reported $C_{max}$ of standard human dosing,
305 or with the endectocide IVM (at 1X $C_{max}$), and combined with RBCs at 40% hematocrit
306 for mosquito feeds. Negative controls included un-spiked blood ("Blood only") or that
307 spiked with drug solvent alone ("Solvent"). Solvents were: ACN:H2O 1:9 + 0.5% FA for
308 AQ, DEAQ, PPQ; ACN 1:1 H2O + 0.1% HCl for SDX; ACN 1:1 H2O + 0.5% FA for PYR
309 (for SP experiments, "Solvent" represents the combination of both SDX and PYR
310 solvents). DMSO (as solvent control for IVM drug) was also tested in AQ and DEAQ
311 feeding experiments. Approximately 50 mosquitoes were fed per group. A-C) Mosquito
312 feeding behavior plotted as percent taking a bloodmeal as percent taking a bloodmeal.
313 Means are plotted with error bars representing SD. D-F) Mosquito survival post drug
314 ingestion plotted in a Kaplan-Meier survival curve (monitored for 14 days). Each graph
315 represents combined data from 3 independent experiments (with 3 data points per
316 condition/time), unless otherwise specified: For AQ and DEAQ feeding and survival,
317 experiments were repeated 4 times, but there were missing data points amounting to
318 only triplicate survival observations for Day 5, 9, 11. For one SP experimental replicate,
319 no mosquitoes fed for the SP1/2X condition (hypothesized due to physical feeder
320 positioning), thus there were only duplicate observations for SP1/2X survival Day 1-14.

321

322 Nor were there consistent differences between control un-spiked versus drug-spiked

323 blood feeds for any compound regarding routine daily behavior and survival, other than
324 for IVM (Fig 2 D-F; S1 Supporting text, S3-S4 Tables). DMSO solvent was also
325 confirmed to have no impact on field mosquito fitness measures when included as a
326 separate control parameter (S3-S4 Tables). Between IVM feeds for all field mosquito
327 experiments, mean 63.0% remained alive on D2 (SD 28.8), 32.1% on D3 (SD 27.7),
328 and 15.8% on D4 (SD 23.4). These overall mortality rates were similar to lab-adapted
329 *An. gambiae* IVM data (mean 70.8% alive on D2 (SD 29.7), 31.5% on D3 (SD 28.8),
330 and 10.6% on D4 (SD 10.4)). However, for the *An. coluzzii* field mosquitoes, mean
331 11.5% remained alive by D7 (SD 21.9) and 3.1% by D14 (SD 4.9) after IVM feed,
332 whereas for the *An. gambiae* lab mosquitoes, <1% remained alive by D6 after IVM feed
333 (D6 mean 0.5% alive (SD 0.9)) (S3 Fig, S3 Table). Differences in overall survival curves
334 comparing IVM-fed *An. gambiae* and *An. coluzzii* mosquitoes were also statistically
335 significant ($p<0.0001$) (S3 Fig, S4 Table). For fertility assessments, field mosquitoes did
336 not routinely lay eggs during each experimental replicate. However, these egg laying
337 deficits also manifested following control un-spiked blood feeds. Of limited available
338 data, no fertility differences were noted upon ingestion of any drug in terms of number of
339 eggs laid per total mosquitoes, nor percent of mosquitoes laying eggs or number of
340 eggs per laying mosquito (S4 Fig and S5-S6 Tables).
341

## 342 DEAQ is absorbed after ingestion and distributed into
## 343 *Anopheles gambiae* circulation and tissues

344 We hypothesized that ingested antimalarials could be absorbed from the midgut and
345 circulate more widely, demonstrating the potential of drug exposure to later parasite

346 stages in development. As a model system to assess this, we assayed the distribution
347 of DEAQ in the mosquito using liquid chromatography with tandem mass spectrometry
348 (LC-MS/MS) following drug-spiked blood feeds. Drug feeding conditions included DEAQ
349 at one-half and twice $C_{max}$ (of 500 ng/ml) as well as IVM at $C_{max}$ (50 ng/ml), with
350 mosquito tissue harvest occurring at 24 hrs and 120 hrs post feed (analyzing drug
351 presence before and after completion of bloodmeal digestion at ~72 hrs). We compared
352 concentrations in 3 sample types: 1) whole mosquitoes, 2) midguts only, and 3)
353 harvested hemolymph. We hypothesized midguts would contain the majority of drug
354 following a bloodmeal, and that any drug presence in hemolymph would be indicative of
355 drug absorption across the midgut into mosquito circulation. Whole mosquitoes or other
356 target compartments were pooled (n=5) and collected in triplicate for each condition,
357 with the overall experiment replicated twice.
358
359 Hemolymph collected via capillary perfusion of the mosquito thorax with PBS was
360 assayed directly, whereas whole mosquitoes and midguts were harvested into an
361 extraction solvent [26] and pulverized prior to mass spectrometry analysis. DEAQ had a
362 limit of quantification of 0.5 ng/ml and was detected in a dose-dependent manner based
363 on feeding at 2X or 1/2X $C_{max}$ at 24 hrs in whole mosquitoes (mean 42.4 ng/ml or 6.7
364 ng/ml, respectively, $p<0.0001$), midguts (mean 33.2 ng/ml or 3.2 ng/ml, respectively,
365 $p=0.0082$), and also hemolymph (mean 0.51 ng/ml for 2X $C_{max}$; undetected at 1/2X $C_{max}$
366 condition). DEAQ was also dose dependently detected based on feeding at either 2X or
367 1/2X $C_{max}$ concentration at 120 hours in all compartments: whole mosquitoes (mean
368 33.1 ng/ml or 5.7 ng/ml, respectively, $p=0.0001$), midguts (mean 4.4 ng/ml or 0.6 ng/ml,

369 respectively), and hemolymph (mean 0.9 ng/ml or 0.2 ng/ml, respectively). Between 24
370 to 120 hrs, DEAQ drug levels decreased in midguts but increased in hemolymph (trends
371 present for both fed concentrations), providing evidence of absorption beyond midgut
372 epithelium and prolonged circulation within hemolymph (Fig 3, Table 1, S7-S8 Tables).
373 IVM had a limit of quantification of 1 ng/ml and was not detected other than in a few
374 samples (detected in all three whole mosquito replicates in one experiment at average
375 of 4.8 ng/ml). However, despite lacking extensive demonstration of biochemical drug
376 absorption for IVM, mosquito behavior changed drastically upon IVM feed resulting in
377 minimal movement and flaccid paralysis, highlighting the technical difficulties in
378 capturing drug detection in individual mosquitoes even when it is clearly present.
379 Harvest at 120 hr was not conducted for IVM because mosquitoes died before that time
380 point was reached.
381
382 **Fig 3. DEAQ is absorbed dose-dependently and detectable beyond midgut**
383 **epithelium up to 5 days following bloodmeal ingestion.**
384 A) Cartoon depiction of mosquito anatomy upon bloodmeal ingestion. B) Mosquitoes
385 were fed 1ɥg/ml or 250 ng/ml DEAQ to represent twice (2X) and half (1/2X) reported
386 $C_{max}$, respectively. Mosquitoes were harvested 24 hrs and 120 hrs post-feed, either
387 collected whole or used for hemolymph collection and midgut dissection. For each
388 condition, 5 of each mosquito sample type were pooled, with triplicates of pooled
389 samples submitted to NorthEast BioLab (Hamden, CT) for LC-MS/MS analysis. Data
390 represents average values from 2 separate experiments. Significant differences
391 highlighted as **** = $p<0.0001$ and ** = $p<0.01$.

392 **Table 1. DEAQ is absorbed and detectable in mosquito compartments up to 5**

393 **days beyond bloodmeal ingestion.**

| **DEAQ detected in mosquitoes after spiked bloodmeal in ng/ml** | | | | |
|---|---|---|---|---|
| **DEAQ fed concentration** (relative to reported $C_{max}$) | **24h post feed** | | **120h post feed** | |
| | **2X** | **1/2X** | **2X** | **1/2X** |
| **Whole mosquitoes** | 42.42 (13.48) | 6.67 (7.17) | 33.14 (8.30) | 5.71 (4.91) |
| **Midgut alone** | 33.22 (28.79) | 3.24 (3.96) | 4.37 (4.06) | 0.57 (0.88) |
| **Hemolymph alone** | 0.51 (0.76) | 0.00 (0.00) | 0.93 (0.83) | 0.21[a] (0.33) |

394 Mosquitoes were fed 1ɥg/ml or 250 ng/ml DEAQ to represent twice (2X) and half (1/2X)

395 reported $C_{max}$, respectively. Mosquitoes were harvested 24 hrs and 120 hrs post-feed,

396 either collected whole or used for hemolymph collection and midgut dissection. For

397 each condition, 5 of each mosquito sample type were pooled, with triplicates of pooled

398 samples submitted to NorthEast BioLab (Hamden, CT) for LC-MS/MS analysis. Data

399 represents average values from 2 separate experiments (with Standard Deviation).

400 [a]The mean reported drug concentration at 120 hours for 1/2 $C_{max}$ condition was lower

401 than the LOQ for hemolymph conditions due to some replicates with no detectable drug.

402

# 403 Discussion

404 *Anopheles* mosquitoes, the vector for malaria transmission, are widely exposed to

405 antimalarials by virtue of feeding on human hosts with long-acting drugs circulating in

406 their blood. We fed *Plasmodium*-uninfected mosquitoes drug-spiked bloodmeals
407 mimicking real-world drug concentrations of the antimalarials AQ, DEAQ, PPQ, and SP.
408 We initially investigated uninfected mosquitoes to determine if drugs had any impact on
409 mosquito viability which could in turn impact parasite transmission dynamics. We did not
410 identify any consistent significant effects of these antimalarials on mosquito feeding
411 behavior, activity levels, fertility, or survival up to 14 days following a drug-spiked blood
412 meal (the time scale required to complete sporogony) in either a lab-adapted *Anopheles*
413 *gambiae* mosquito strain or a field-maintained *Anopheles coluzzii* strain. Using DEAQ
414 as a model due to its widespread use in SMC, we next assessed whether we could
415 demonstrate absorption of long-acting antimalarials via blood-feeds using LC-MS/MS.
416 We were able to detect drug present in whole mosquitoes, as well as within isolated
417 midguts and hemolymph, 5 days post ingestion in a dose-dependent manner. The fact
418 that drug levels decreased over time in midguts, while increasing in hemolymph, further
419 supports that drugs present in mosquito bloodmeals can be absorbed beyond the
420 midgut into the mosquito circulation and remain present in the circulation days after
421 bloodmeals have been digested. To our knowledge, this is the first report of human
422 antimalarial biochemical quantitative detection within *Anopheles* mosquito tissues
423 following bloodmeal exposure.
424
425 With evidence that human antimalarials can be absorbed and persist in mosquito
426 hemolymph, we hypothesize that drugs present in the bloodmeal have the potential to
427 impact malaria parasite development and resistance selection during sporogony.
428 Antimalarial resistance potentiation during mosquito stages has largely been overlooked

429 compared to studies focused on the human blood stage. One area that has been
430 examined is whether certain antibiotics can impact parasite development in the
431 mosquito when ingested via blood meal [30,31]. The authors investigated penicillin-
432 streptomycin, doxycycline, azithromycin, and trimethoprim-sulfamethoxazole. In some
433 instances, antibiotics in feeds enhanced susceptibility of *An. gambiae* to *P. falciparum*
434 infection (antibiotic exposure occurring at time of mosquito infection) and positively
435 impacted vector lifespan and fecundity (postulated to be from mosquito gut microbiota
436 disturbances, or possibly reduced levels of mosquito innate immune activation post
437 blood feed) [30], though effects varied by antibiotic [31]. In particular, trimethoprim-
438 sulfamethoxazole, an anti-folate similar to SP, reduced *P. falciparum* oocyst intensity.
439 Recent work also has shown exposing mosquitoes to atovaquone starting at 48 hrs
440 post-infection (via spiked sugar meal) significantly reduces oocyst size and sporozoite
441 counts [29].
442
443 Antimalarial resistance develops primarily during symptomatic human blood-stage
444 infection when replication rates and drug pressure are highest [11]. However, mosquito-
445 level drug exposure could still impact already-developing parasites and contribute to
446 strain selection at different points during sporogony. Studies investigating bottlenecks
447 during sporogony emphasize the relevance of parasite oocyst and sporozoite numbers
448 on overall transmission success, yet the specific determinants that impact parasite
449 progression through vector-stage bottlenecks remain poorly understood [49,50]. Some
450 research assessing infection of mosquitoes fed on gametocyte carriers with multiclonal
451 infections has suggested both preferential transmission of minority clones to mosquitoes

452 and propagation of minority clones through the mosquito, with significant implications for
453 potential onward transmission of minority alleles including those that confer drug
454 resistance [51,52].
455
456 While the DEAQ concentration we detected was low, our methods would underestimate
457 drug levels given significantly diluted analyte and limited sampling times. The reported
458 DEAQ $IC_{50}$ of ~60 nM [53,54] (~24 ng/ml) is on par with levels we detected in whole
459 mosquitoes and midguts at 24 hrs and whole mosquitoes at 120 hrs. Oocysts,
460 developing between the mosquito midgut epithelium and basal lamina [55], could
461 conceivably be exposed to ingested drug either directly via midgut bloodmeal contents
462 (noting additional feeds stress and damage the midgut epithelial layer [21]) or via drug
463 absorption into hemolymph (and subsequent tissue distribution). While oocysts are
464 often portrayed as relatively “walled off” and thus potentially more protected from drug
465 activity, atovaquone studies show that oocyst development can be impacted by either
466 tarsal surface exposure (tissue absorption) or feeding [29]. Furthermore, drug present in
467 midgut or circulation could impact pre-oocyst stages or sporozoites that travel via
468 hemolymph to the salivary glands. We offer strong proof-of-concept data that support
469 extensive drug distribution in mosquitoes for prolonged periods. Given 30-50% of some
470 communities may be taking long-acting antimalarials, even rare selection events or low-
471 level drug activity in mosquitoes could have large implications for malaria transmission
472 dynamics in endemic areas.
473

474 There are several limitations to our present study. Although we only tested two
475 *Plasmodium*-susceptible *Anopheles* species, we did compare lab and field mosquitoes,
476 providing further evidence that the three antimalarials studied do not have an impact on
477 mosquito fitness and viability. The IVM survival data highlights the relevance of testing
478 field mosquitoes, given some were able to resist the endectocidal effect of IVM control
479 feeds compared to lab mosquitoes (where <1% remained alive by D6). Future work
480 would be bolstered by increasing the replicates/sample sizes from our field study, as we
481 were not able to collect more fertility data given that mosquitoes did not lay eggs
482 following feeds from some blood bank samples. We suspect this may be due to stored
483 blood bank products suffering from breakdown of nutrients required for egg
484 development. Despite this limitation, when egg laying did result from a feed, our data
485 were consistent between control un-spiked vs. spiked drug feeds.
486
487 While some statistically significant differences in feeding behavior were present in
488 alternative individual chi-squared analyses between individual spiked bloodmeal
489 conditions in comparison to un-spiked blood or matching solvent-spiked blood, these
490 differences were not consistent in direction between drug concentrations, mosquito
491 species, nor between the same control conditions (e.g. IVM feeds) across different
492 experimental groups. Thus, we believe they are more reflective of general variability
493 between bloodmeal ingestion during any given experiment – particularly with the field
494 mosquitoes that are less adapted to artificial membrane feeding. Similarly, isolated
495 survival curves with significant differences are likely more reflective of one-off
496 technically significant results that arise in the myriad multiple comparisons done within

497 each drug or solvent feeding group and mosquito species, as there was no consistency
498 in significance across drug concentration and mosquito species. Minimally, our results
499 show that drug presence did not negatively impact survival, with most of the significant
500 curve differences showing the lower concentration of drug or drug-absent blood only
501 condition had reduced survival.
502
503 We focused our initial drug detection analyses on DEAQ given its widespread use in
504 both treatment and prevention, with the primary goals of determining whether we could
505 1) detect drug in various mosquito compartments, and 2) observe any concentration
506 changes over time. Our time- and labor-intensive design was successful in achieving
507 these objectives, and we are not surprised that there was variability in terms of the
508 number of samples with detectable drug, particularly since concentrations were often
509 near the limit of LC-MS/MS quantification. This is true for IVM, which was undetectable
510 in our study with a LOQ of 1 ng/ml. However, IVM was clearly absorbed, as evidenced
511 by mosquito death following ingestion. Given these experimental limitations, the fact
512 that we could successfully detect DEAQ in individual mosquitoes days after ingestion,
513 even in isolated hemolymph, supports that antimalarial absorption occurs, and can
514 potentially interface with different stages of sporogonic development. Furthermore, our
515 DEAQ detection results were highly specific (with no drug detection in any negative
516 controls fed solvent-spiked blood or unaltered blood) and concentration dependent (in
517 terms of initial fed drug amount).
518
519 Having demonstrated mosquito drug exposure occurs but does not itself negatively

impact the vector, the next question will be investigating the impact of antimalarial exposure on infected mosquito oocyst and sporozoite output, as well as assessing whether drugs could impact the success of strains developing in the mosquito, particularly in mixed infections where less resistant strains may have a developmental advantage. Given rapidly expanding drug resistance to artemisinin based antimalarials now in Africa [4], concern about partner drug resistance that could contribute to failure of first-line treatment, and stagnating progress on reducing the global malaria burden in recent years, the malaria community needs to consider all avenues of possible contribution to drug-resistance spread. This includes the possibility that human antimalarials could impact vector stage transmission dynamics. Better understanding of mosquito drug exposure and its impact could also help in developing strategies to target the parasite within the mosquito.

# Materials and methods

## Mosquito colonies

Two species of mosquitoes were used in this study: 1) *An. gambiae* “G3” strain, a highly lab-adapted, insecticide-susceptible strain of African origin (obtained from the Catteruccia lab, Harvard University) reared at Yale lab facilities in New Haven, CT under standard conditions (27 ± 2°C, 70% humidity, 12 hour light cycle with access to 10% glucose solution); referred to as “lab mosquitoes;” and 2) *Anopheles coluzzii* outbred mosquitoes (established in 2023 and repeatedly replenished with F1 from wild-caught female mosquitoes collected in Bama, Burkina Faso (coordinates 12.0395° N, 4.4010° W; ~60km north of Bobo-Dioulasso); field-collected female mosquitoes morphologically

543 identified as belonging to the *An. gambiae* complex further identified by PCR before
544 pooling) and maintained either in the Institut de Recherche en Sciences de la Santé
545 (IRSS) laboratory in Bobo Dioulasso under standard conditions (27 ± 2°C, 70%
546 humidity, 12 hour light cycle with access to 10% glucose solution) or in the Bama
547 malaria sphere under semi-natural conditions (exposed to all environmental factors
548 including temperature and relative humidity). These mosquitoes (maintained in either
549 Bama malaria sphere or Bobo-Dioulasso lab conditions) are collectively referred to as
550 "field mosquitoes."
551

552 ## Drug-spiked blood feeds

553 Fresh human blood was routinely obtained from healthy O+ donors in New Haven, CT
554 or via hospital blood bank in Bobo-Dioulasso, Burkina Faso. For human donors at Yale,
555 plasma was removed and packed RBCs were separated and washed with un-
556 supplemented RPMI media. Blood products in Burkina Faso were obtained as packed
557 RBCs and used as is. Drugs of interest were dissolved into relevant solvent at 10mg/ml
558 and stored at -20°C. Briefly, amodiaquine (AQ; Sigma), desethylamodiaquine (DEAQ;
559 Cayman), and piperaquine (PPQ; Sigma) were dissolved into 1:9 v/v acetonitrile:H2O
560 with 0.5% formic acid; sulfadoxine (SDX; Cayman) was dissolved into 1:1 v/v
561 acetonitrile:H2O with 0.1% hydrochloric acid; pyrimethamine (PYR: Cayman) was
562 dissolved into 1:1 v/v acetonitrile:H2O with 0.5% formic acid; and ivermectin (IVM;
563 Sigma) was dissolved into DMSO. Drug stocks were diluted into either plasma (Yale
564 donors from above) or commercial non-immune AB serum (Sigma; used for Burkinabé
565 experiments) to achieve physiologic concentrations in the range of one-half to two times

566 the average reported $C_{max}$ after a standard human oral dose as per available literature
567 ($C_{max}$ approximated as 20 ng/ml for AQ [42], 500 ng/ml for DEAQ [42], 300 ng/ml for
568 PPQ [41,43,44], 100 ɥg/ml for SDX [40,45], 400 ng/ml for PYR [40,45], 50 ng/ml for IVM
569 [46]). Drug-spiked plasma/serum was then combined with packed RBCs at 40%
570 hematocrit and fed for up to 60 minutes to 5-10 day old *Anopheles* mosquitoes via
571 standard membrane feeding practice with pre-warmed glass feeders and parafilm
572 membrane.
573

## 574 Mosquito viability assessments

575 50 female mosquitoes per condition were placed in individual containers for drug-spiked
576 blood feeds, after which fed mosquitoes were manually separated and maintained at
577 standard conditions. For all experiments, mosquitoes were only offered one singular
578 feeding opportunity and never refed. Feeding behavior was determined by the
579 percentage of female mosquitoes present that imbibed a bloodmeal. Post feeding,
580 viability was monitored for each condition for up to 14 days, to span the typical amount
581 of time sporozoites develop in a mosquito. Daily mosquito behavior was also monitored
582 by gently blowing on the cartons of mosquitoes and assessing flight activity. Finally, 2
583 days after feeding, 5 randomly selected individual mosquitoes per condition were placed
584 in individual containers for forced egg laying, with the number of eggs per mosquito
585 recorded, as well as the number of laying mosquitoes. (Fertility assessments did not
586 take place for the ivermectin condition because by this time post feed, these mosquitoes
587 were notably unhealthy and not able to withstand forced egg laying conditions.)
588 Experiments were repeated in triplicate. Statistical comparisons between mosquito

589 fertility were assessed via ordinary one-way ANOVA with Dunnett's multiple
590 comparisons test to compare each condition to "blood only" control (performed in
591 GraphPad Prism version 10.5.0). Statistical comparisons between mosquito feeding
592 behavior were assessed both via ordinary one-way ANOVA of percent of mosquitoes
593 feeding with Dunnett's multiple comparisons test to compare each condition to "blood
594 only" or control, as well as using contingency table analyses of total fed and unfed
595 mosquitoes combining the data from multiple experiments for each drug feeding group
596 and comparing a given experimental condition to the control "blood only" or "solvent"
597 condition (both performed in GraphPad Prism version 10.5.0). Survival was assessed
598 via stratified log-rank test of Kaplan Meier analysis combining the data from multiple
599 experiments for each drug feeding group and using the Tukey-Kramer adjustment for
600 multiple comparisons (performed in SAS 9.4, Cary, NC).
601

## 602 Biochemical drug detection in the mosquito

603 We analyzed drug absorption within individual mosquitoes using IVM (at 24 hours post
604 feed) and DEAQ (at 24 hours and 120 hours post feed). 120 mosquitoes were fed per
605 condition (control blood only, IVM 1X, ACN:$H_2O$ 1:9 + 0.5% FA solvent, DMSO solvent,
606 DEAQ 2X, DEAQ 1/2X). Fed mosquitoes were manually separated and maintained at
607 standard conditions. Per condition, 5 mosquitoes were pooled into 100 µl "extraction
608 solvent" (70% MeOH + 0.1% formic acid, per others' reported methods [26]), 5 mosquito
609 midguts were harvested into 50 µl of extraction solvent, and hemolymph was pooled
610 from 5 mosquitoes; these were all collected in triplicate. To collect hemolymph,
611 mosquitoes were immobilized on ice under a dissecting microscope. The last segment

612 of the abdomen was removed. A glass capillary tube made to fine needle point attached
613 to 1 mm diameter silicone tubing and a 100 µl Hamilton syringe mounted on an
614 automated microdispenser (PB600 Repeating Syringe Dispenser, Hamilton) filled with
615 PBS was used to puncture the thorax underneath the wings, with 2 µl of PBS injected in
616 increments. Resultant hemolymph expelled out of the dissected abdomen was collected
617 via p10 pipette (approximately 2-5 µl of hemolymph diluted into PBS collected per
618 mosquito). Hemolymph-PBS solutions were not further processed other than high speed
619 centrifugation at 16.1g for 10 minutes with supernatant submitted for analysis. Whole
620 mosquitoes and midguts were pulverized using the pellet pestle grinder (DWK Life
621 Sciences), vortexed for 30 sec, sonicated for 10 min, and centrifuged at 16.1g for 10
622 min with supernatants submitted for LC-MS/MS drug detection (similar to described
623 [26]). Drug detection was performed by NorthEast BioLabs (Hamden, CT) with
624 appropriate standard calibration using control matrix consisting of sugar-fed whole
625 mosquitoes in extraction solvent. Reported limits of quantification were 1 ng/ml for
626 ivermectin and 0.5 ng/ml for desethylamodiaquine. Data represents results from
627 triplicate pooled samples for each harvest parameter, averaged between two identical
628 repeated experiments. Statistical comparisons between drug levels detected in different
629 mosquito compartments were assessed via one-way ANOVA followed by pairwise
630 comparisons of interest using Holm-Šídák's multiple comparisons test (calculated using
631 GraphPad Prism version 10.5.0).
632

633 ## Ethics statement

634 Human subjects work involving use of blood from U.S. donors was approved by the Yale

University Institutional Review Board (IRB 2000034487). Formal verbal consent was obtained for all subjects involved. Blood from Burkina Faso donors was obtained via local hospital blood bank in Bobo-Dioulasso, Burkina Faso (IRB exempt de-identified blood products).


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846

# 847 Supporting information

848 **S1 Supporting text.**

849

850 **S1 Fig. Solvents do not significantly impact lab-adapted *An. gambiae* behavior,**
851 **viability, or fertility.** Solvents used to dissolve drugs AQ, DEAQ, PPQ, SDX, PYR, and
852 IVM were diluted into plasma at 1:833 dilution and combined with RBCs at 40%
853 hematocrit for mosquito feeds. Un-spiked blood ("Blood only") served as control.
854 Abbreviations include: ACN – acetonitrile, used at either 1:1 or 1:9 v/v with $H_2O$; FA –
855 formic acid; HCl – hydrochloric acid. Approximately 50 mosquitoes were fed per group.
856 A) Mosquito feeding behavior plotted as percent taking a bloodmeal. B) Mosquito
857 survival post solvent ingestion plotted in a Kaplan-Meier survival curve (monitored for 14
858 days). C) Mosquito fertility assessed by total eggs laid per condition (all mosquitoes
859 monitored communally per experiment). Data are from experiments performed in
860 duplicate (except for only single survival data points available for D9 and D11 ACN:$H_2O$
861 1:1 + HCl 0.1% and HCl 0.1% conditions). In A and C, means are plotted with error bars
862 representing SD.

863

864 **S2 Fig. Tested antimalarials do not significantly impact percent of lab-adapted *An.***
865 ***gambiae* mosquitoes laying eggs.** Human plasma was spiked with antimalarials AQ,
866 DEAQ, PPQ, or SP at levels ranging from one half (1/2X) to twice (2X) the average
867 reported $C_{max}$ of standard human dosing and combined with RBCs at 40% hematocrit
868 for mosquito feeds. Negative controls included un-spiked blood ("Blood only") or blood
869 spiked with drug solvent alone ("Solvent"). Solvents were: ACN:$H_2O$ 1:9 + 0.5% FA for
870 AQ, DEAQ, PPQ; ACN 1:1 $H_2O$ + 0.1% HCl for SDX; ACN 1:1 $H_2O$ + 0.5% FA for PYR
871 (for SP experiments, "Solvent" represents the combination of both SDX and PYR
872 solvents). Approximately 50 mosquitoes were fed per group, measuring effect of A) AQ
873 and DEAQ, B) PPQ, and C) SP on mosquito fertility as measured by percent
874 mosquitoes laying eggs (of n=5 mosquitoes monitored per experiment). Each graph
875 represents combined data from 3 independent experiments. Means are plotted with
876 error bars representing SD. There were no significant differences in percent of egg
877 laying mosquitoes between groups.

878

879 **S3 Fig. Reduced IVM killing effects in field-derived *An. coluzzii* versus lab-derived**
880 ***An. gambiae* mosquitoes.** Human plasma was spiked with the endectocide IVM (at 1X
881 $C_{max}$ of standard human dosing) and combined with RBCs at 40% hematocrit for
882 mosquito feeds. Un-spiked blood ("Blood only") served as control. Approximately 50
883 mosquitoes were fed per group. Mosquito survival post drug ingestion plotted in a
884 Kaplan-Meier survival curve (monitored for 14 days). Data here is combined from
885 multiple available independent feeding experiments (9 for *An. gambiae* and 10 for *An.*

886 *coluzzii*, except the “Blood only” condition for *An. gambiae* had 8 data points for D 10,
887 11, 12, 14, and 6 data points for D9, 13; and both conditions for *An. coluzzii* had 9 data
888 points for D 5, 9, 11). There were no significant differences in “Blood only” survival
889 between lab and field mosquitoes, however for “IVM 1X” the *An. coluzzii* mosquitoes
890 demonstrated significantly improved survival compared to *An. gambiae* mosquitoes
891 ($p<0.0001$).
892
893 **S4 Fig. Tested antimalarials do not significantly impact field-adapted *An. coluzzii***
894 **fertility.** Human serum was spiked with antimalarials AQ, DEAQ, PPQ, or SP at levels
895 ranging from one half (1/2X) to twice (2X) the average reported $C_{max}$ of standard human
896 dosing and combined with RBCs at 40% hematocrit for mosquito feeds. Negative
897 controls included un-spiked blood (“Blood only”) or blood spiked with drug solvent alone
898 (“Solvent”). Solvents were: ACN:$H_2O$ 1:9 + 0.5% FA for AQ, DEAQ, PPQ; ACN 1:1 $H_2O$
899 + 0.1% HCl for SDX; ACN 1:1 $H_2O$ + 0.5% FA for PYR (for SP experiments, “Solvent”
900 represents the combination of both SDX and PYR solvents). Approximately 50
901 mosquitoes were fed per group. Mosquito fertility (of n=5 mosquitoes monitored per
902 experiment) is depicted both by total eggs laid per condition (A-C) and percent of
903 mosquitoes laying eggs (D-F). Each graph represents combined data from 3
904 independent experiments, however for many experimental replicates, mosquitoes did
905 not routinely lay eggs – for AQ and DEAQ feeds, there was only egg laying data from 2
906 replicates (and the DEAQ 2X-fed group from one additional replicate); for PPQ feeds,
907 there was only egg laying data from 1 replicate (and the PPQ 2X-fed group from one
908 additional replicate). Means are plotted with error bars representing SD.

909

910 **S1 Table. Feeding rates for lab-derived *An. gambiae* and field-derived *An. coluzzii***
911 **offered bloodmeals spiked with solvents and tested antimalarials.** Data represents
912 the percent of female mosquitoes that fed (abdomen with visible bloodmeal) under each
913 condition, reported as the mean between experimental replicates (and Standard
914 Deviation) (two replicates for initial solvent control feeding; three replicates for all drug
915 feeding experiments). Plasma was spiked with solvents DMSO, ACN:$H_2O$ 1:1 v/v +
916 0.5% FA, ACN:$H_2O$ 1:9 v/v + 0.5% FA, ACN:$H_2O$ 1:1, FA 0.5%, ACN:$H_2O$ 1:1 + 0.1%
917 HCl, and 0.1% HCl all used at 1:833 dilution in plasma and mixed with RBCs to achieve
918 40% hematocrit in initial control experiments using lab-derived *An. gambiae*. In further
919 experiments with both lab *An. gambiae* and field *An. coluzzii*, plasma or serum was
920 spiked with the antimalarials AQ, DEAQ, PPQ, or SP at levels ranging from one half
921 (1/2X) to twice (2X) the average reported $C_{max}$ of standard human dosing, or with the
922 endectocide IVM (at 1X $C_{max}$), and combined with RBCs at 40% hematocrit for mosquito
923 feeds. Negative controls included un-spiked blood ("Blood only") or for the drug feeding
924 experiments, blood spiked with the relevant drug solvent alone ("Solvent"). Solvents
925 used in the drug feeding experiments were: ACN:$H_2O$ 1:9 + 0.5% FA for AQ, DEAQ,
926 PPQ; ACN:$H_2O$ 1:1 + 0.1% HCl for SDX; ACN:$H_2O$ 1:1 + 0.5% FA for PYR (for SP
927 experiments, "Solvent" represents the combination of both SDX and PYR solvents).
928 Approximately 50 mosquitoes were fed per group.

929

930 **S2 Table. Raw feeding data and contingency analyses for lab-derived *An. gambiae***
931 **and field-derived *An. coluzzii* offered bloodmeals spiked with solvents and tested**

932 **antimalarials.** Data represents the number of female mosquitoes that fed (abdomen
933 with visible bloodmeal) or did not feed under each condition, reported as the total
934 between experimental replicates (two replicates for initial solvent control feeding; three
935 replicates for all drug feeding experiments). Contingency analyses comparing the rates
936 of fed to unfed mosquitoes for each experimental condition are included against the
937 blood only or relevant solvent controls, as well as for total lab *An. gambiae* and field *An.*
938 *coluzzii* feeding rates under all conditions (reported as p values, italicized if <0.05 with
939 direction of significance stated). Plasma was spiked with solvents DMSO, ACN:$H_2O$ 1:1
940 v/v + 0.5% FA, ACN:$H_2O$ 1:9 v/v + 0.5% FA, ACN:$H_2O$ 1:1, FA 0.5%, ACN:$H_2O$ 1:1 +
941 0.1% HCl, and 0.1% HCl all used at 1:833 dilution in plasma and mixed with RBCs to
942 achieve 40% hematocrit in initial control experiments using lab-derived *An. gambiae*. In
943 further experiments with both lab *An. gambiae* and field *An. coluzzii*, plasma was spiked
944 with the antimalarials AQ, DEAQ, PPQ, or SP at levels ranging from one half (1/2X) to
945 twice (2X) the average reported $C_{max}$ of standard human dosing, or with the endectocide
946 IVM (at 1X $C_{max}$), and combined with RBCs at 40% hematocrit for mosquito feeds.
947 Negative controls included un-spiked blood ("Blood only") or for the drug feeding
948 experiments, blood spiked with the relevant drug solvent alone ("Solvent"). Solvents
949 used in the drug feeding experiments were: ACN:$H_2O$ 1:9 + 0.5% FA for AQ, DEAQ,
950 PPQ; ACN:$H_2O$ 1:1 + 0.1% HCl for SDX; ACN:$H_2O$ 1:1 + 0.5% FA for PYR (for SP
951 experiments, "Solvent" represents the combination of both SDX and PYR solvents).
952 Approximately 50 mosquitoes were fed per group.
953

954 **S3 Table. Percent survival of lab-derived *An. gambiae* and field-derived *An.***
955 ***coluzzii* following ingestion of bloodmeal spiked with solvents and tested**
956 **antimalarials.** Data represents percent survival of mosquitoes (approximately n=50 fed;
957 unfed removed) over time after feeding on bloodmeals spiked with solvents and drugs
958 of interest. Data are from 3 experimental replicates, with individual data for each
959 time/condition shown, unless otherwise indicated (blank for few times/conditions when
960 data was not captured; only 2 replicates for initial solvent control experiments; 4
961 replicates for *An. coluzzii* AQ/DEAQ feeds; 9 replicates combining all *An. gambiae* IVM
962 feeding data and 10 replicates combining all *An. coluzzii* IVM feeding data). Means (and
963 Standard Deviation) are also shown for each time/condition. Plasma was spiked with
964 solvents DMSO, ACN:$H_2O$ 1:1 v/v + 0.5% FA, ACN:$H_2O$ 1:9 v/v + 0.5% FA, ACN:$H_2O$
965 1:1, FA 0.5%, ACN:$H_2O$ 1:1 + 0.1% HCl, and 0.1% HCl all used at 1:833 dilution in
966 plasma and mixed with RBCs to achieve 40% hematocrit in initial control experiments
967 using lab-derived *An. gambiae*. In further experiments with both lab *An. gambiae* and
968 field *An. coluzzii*, plasma was spiked with the antimalarials AQ, DEAQ, PPQ, or SP at
969 levels ranging from one half (1/2X) to twice (2X) the average reported $C_{max}$ of standard
970 human dosing, or with the endectocide IVM (at 1X $C_{max}$), and combined with RBCs at
971 40% hematocrit for mosquito feeds. Negative controls included un-spiked blood ("Blood
972 only") or for the drug feeding experiments, blood spiked with the relevant drug solvent
973 alone ("Solvent"). Solvents used in the drug feeding experiments were: ACN:$H_2O$ 1:9 +
974 0.5% FA for AQ, DEAQ, PPQ; ACN:$H_2O$ 1:1 + 0.1% HCl for SDX; ACN:$H_2O$ 1:1 + 0.5%
975 FA for PYR (for SP experiments, "Solvent" represents the combination of both SDX and
976 PYR solvents).

977

978 **S4 Table. Survival curve comparisons for lab-derived *An. gambiae* and field-**
979 **derived *An. coluzzii* following ingestion of bloodmeal spiked with solvents and**
980 **tested antimalarials.** Data represents Kaplan-Meier survival curve comparisons
981 between specified groups, with Chi square results and p values (unadjusted and
982 adjusted for multiple comparisons using Tukey-Kramer post-hoc analyses). Significant
983 adjusted p values <0.05 are italicized for comparisons not involving IVM, with the
984 direction of significance noted. Survival curves for each group are from 3 experimental
985 replicates (other than only 2 replicates for initial solvent control experiments; 4
986 replicates for An. coluzzii AQ/DEAQ feeds). Data comparing survival curves for all blood
987 only controls and IVM 1X feeds in *An. gambiae* versus *An. coluzzii* mosquitoes is also
988 included. Plasma was spiked with solvents DMSO, ACN:$H_2O$ 1:1 v/v + 0.5% FA,
989 ACN:$H_2O$ 1:9 v/v + 0.5% FA, ACN:$H_2O$ 1:1, FA 0.5%, ACN:$H_2O$ 1:1 + 0.1% HCl, and
990 0.1% HCl all used at 1:833 dilution in plasma and mixed with RBCs to achieve 40%
991 hematocrit in initial control experiments using lab-derived *An. gambiae*. In further
992 experiments with both lab *An. gambiae* and field *An. coluzzii*, plasma was spiked with
993 the antimalarials AQ, DEAQ, PPQ, or SP at levels ranging from one half (1/2X) to twice
994 (2X) the average reported $C_{max}$ of standard human dosing, or with the endectocide IVM
995 (at 1X $C_{max}$), and combined with RBCs at 40% hematocrit for mosquito feeds. Negative
996 controls included un-spiked blood ("Blood only") or for the drug feeding experiments,
997 blood spiked with the relevant drug solvent alone ("Solvent"). Solvents used in the drug
998 feeding experiments were: ACN:$H_2O$ 1:9 + 0.5% FA for AQ, DEAQ, PPQ; ACN:$H_2O$ 1:1

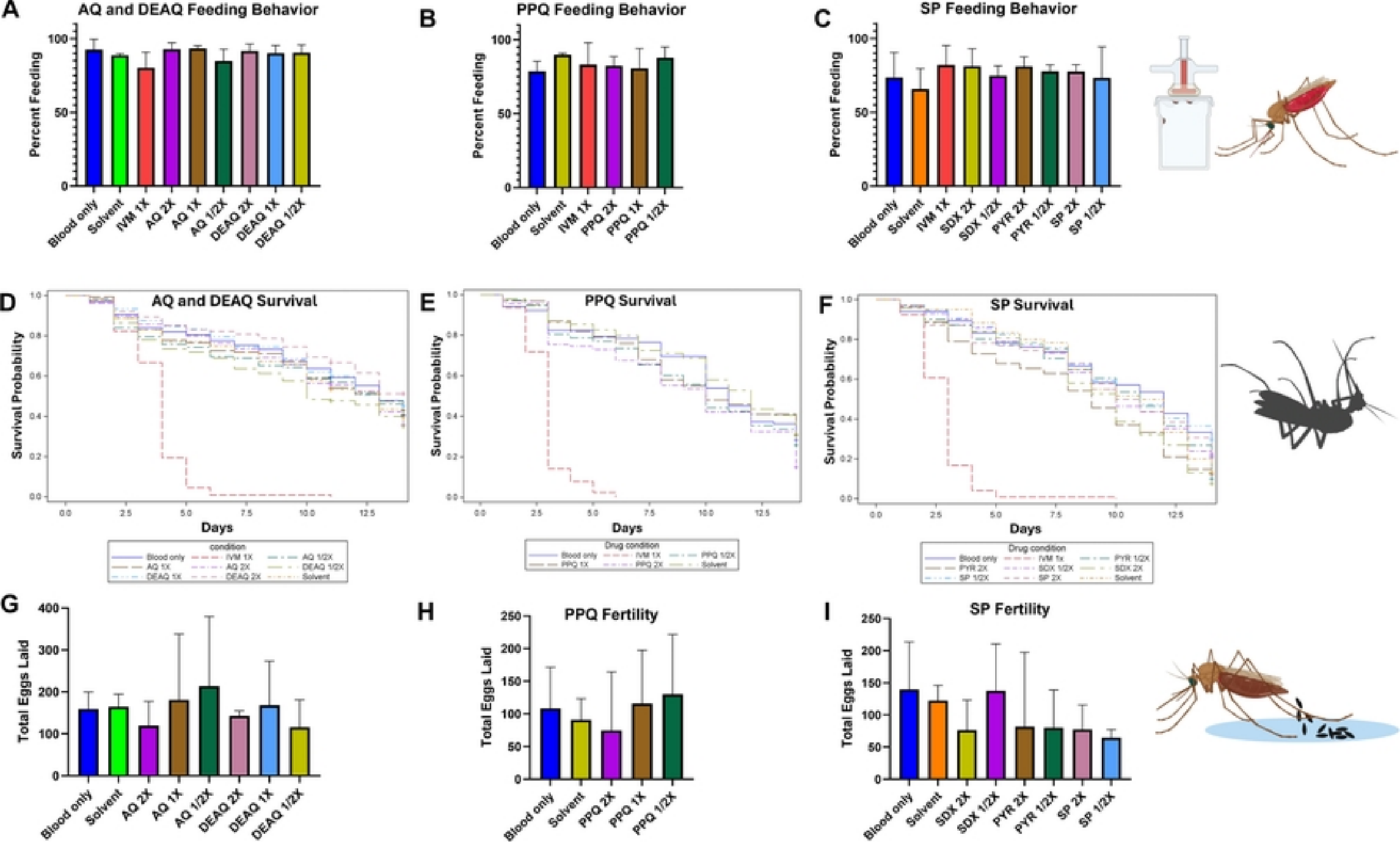
999 + 0.1% HCl for SDX; ACN:$H_2O$ 1:1 + 0.5% FA for PYR (for SP experiments, “Solvent”
1000 represents the combination of both SDX and PYR solvents).
1001
1002 **S5 Table. *An. gambiae* and *An. coluzzii* mosquito fertility following ingestion of**
1003 **bloodmeal spiked with solvents and tested antimalarials.** For initial solvent control
1004 feeds in *An. gambiae*, data includes the total number of eggs laid and eggs laid per
1005 mosquito (all fed mosquitoes monitored communally for egg laying; percent of
1006 mosquitoes laying eggs unknown) from two experimental replicates. For all further drug
1007 feeds, data includes the total eggs laid, number of eggs laid per mosquito, and percent
1008 of mosquitoes laying eggs (n=5 mosquitoes monitored in egg laying conditions)
1009 following bloodmeal ingestion under each condition between experimental replicates
1010 (three replicates for *An. gambiae* and *An. coluzzii* DEAQ and SP feeds; 2 replicates for
1011 *An. coluzzii* PPQ feeds excluding further replicates from analyses where no mosquitoes
1012 laid eggs). Data is reported as the mean (with Standard Deviation). Plasma was spiked
1013 with solvents DMSO, ACN:$H_2O$ 1:1 v/v + 0.5% FA, ACN:$H_2O$ 1:9 v/v + 0.5% FA,
1014 ACN:$H_2O$ 1:1, FA 0.5%, ACN:$H_2O$ 1:1 + 0.1% HCl, and 0.1% HCl all used at 1:833
1015 dilution in plasma and mixed with RBCs to achieve 40% hematocrit in initial control
1016 experiments using lab-derived *An. gambiae*. In further experiments with both lab *An.*
1017 *gambiae* and field *An. coluzzii*, plasma was spiked with the antimalarials AQ, DEAQ,
1018 PPQ, or SP at levels ranging from one half (1/2X) to twice (2X) the average reported
1019 $C_{max}$ of standard human dosing, and combined with RBCs at 40% hematocrit for
1020 mosquito feeds. Negative controls included un-spiked blood (“Blood only”) or for the
1021 drug feeding experiments, blood spiked with the relevant drug solvent alone (“Solvent”).

1022 Solvents used in the drug feeding experiments were: ACN:$H_2O$ 1:9 + 0.5% FA for AQ,
1023 DEAQ, PPQ; ACN:$H_2O$ 1:1 + 0.1% HCl for SDX; ACN:$H_2O$ 1:1 + 0.5% FA for PYR (for
1024 SP experiments, "Solvent" represents the combination of both SDX and PYR solvents).
1025

1026 **S6 Table. Lab-derived *An. gambiae* and field-derived *An. coluzzii* mosquito egg**
1027 **laying data following ingestion of bloodmeal spiked with tested antimalarials.**
1028 Data shown as the number of eggs laid per mosquito (n=5 mosquitoes monitored in
1029 individual egg laying conditions) following bloodmeal ingestion under each condition in
1030 three experimental replicates per group. Plasma was spiked with the antimalarials AQ,
1031 DEAQ, PPQ, or SP at levels ranging from one half (1/2X) to twice (2X) the average
1032 reported $C_{max}$ of standard human dosing and combined with RBCs at 40% hematocrit
1033 for mosquito feeds. Negative controls included un-spiked blood ("Blood only"), or for the
1034 drug feeding experiments, blood spiked with the relevant drug solvent alone ("Solvent")
1035 (DMSO also included as a separate solvent in *An. coluzzii* AQ/DEAQ feeds). Solvents
1036 used in the drug feeding experiments were: ACN:$H_2O$ 1:9 + 0.5% FA for AQ, DEAQ,
1037 PPQ; ACN:$H_2O$ 1:1 + 0.1% HCl for SDX; ACN:$H_2O$ 1:1 + 0.5% FA for PYR (for SP
1038 experiments, "Solvent" represents the combination of both SDX and PYR solvents).
1039

1040 **S7 Table. Drug detection in isolated *An. gambiae* mosquito compartments.**
1041 Mosquitoes were fed 1ɥg/ml or 250 ng/ml DEAQ to represent twice (2X) and half (1/2X)
1042 reported $C_{max}$, respectively, or 50 ng/ml IVM to represent 1X reported Cmax. Controls
1043 included un-spiked blood (Blood only), and solvent-spiked blood (ACN:$H_2O$ 1:9 + 0.5%
1044 FA for DEAQ; DMSO for IVM). Mosquitoes were harvested 24 hrs (all conditions) and

1045 120 hrs (Blood only, ACN+FA, DEAQ 2X, DEAQ 1/2X) post-feed, either collected whole
1046 or used for hemolymph collection and midgut dissection. For each condition, 5 of each
1047 mosquito sample type were pooled, with triplicates of pooled samples submitted to
1048 NorthEast BioLab (Hamden, CT) for LC-MS/MS analysis. All data from 2 separate
1049 experiments is shown. B=blank (no drug detected).
1050
1051 **S8 Table. Drug detection comparisons in isolated *An. gambiae* mosquito**
1052 **compartments over time.** Mosquitoes were fed 1 ɥg/ml or 250 ng/ml DEAQ to
1053 represent twice (2X) and half (1/2X) reported $C_{max}$, respectively, or 50 ng/ml IVM to
1054 represent 1X reported $C_{max}$. Controls included un-spiked blood (Blood only), and
1055 solvent-spiked blood (ACN:$H_2O$ 1:9 + 0.5% FA for DEAQ; DMSO for IVM). Mosquitoes
1056 were harvested 24 hrs (all conditions) and 120 hrs (Blood only, ACN:$H_2O$ 1:9 + 0.5%
1057 FA, DEAQ 2X, DEAQ 1/2X) post-feed, either collected whole or used for hemolymph
1058 collection and midgut dissection. For each condition, 5 of each mosquito sample type
1059 were pooled, with triplicates of pooled samples submitted to NorthEast BioLab
1060 (Hamden, CT) for LC-MS/MS analysis. Data is from 2 separate experiments. Results
1061 are listed for one-way ANOVA analyzing drug levels detected in whole mosquitoes,
1062 midguts, and hemolymph with pairwise comparisons of conditions of interest over time.
1063 Significant p values ($p<0.05$) are italicized.


A
AQ and DEAQ Feeding Behavior
Percent Feeding
Blood only
Solvent
IVM 1X
AQ 2X
AQ 1X
AQ 1/2X
DEAQ 2X
DEAQ 1X
DEAQ 1/2X
B
PPQ Feeding Behavior
Percent Feeding
Blood only
Solvent
IVM 1X
PPQ 2X
PPQ 1X
PPQ 1/2X
C
SP Feeding Behavior
Percent Feeding
Blood only
Solvent
IVM 1X
SDX 2X
SDX 1/2X
PYR 2X
PYR 1/2X
SP 2X
SP 1/2X
D
AQ and DEAQ Survival
Survival Probability
Days
condition
Blood only
IVM 1X
AQ 1/2X
AQ 1X
AQ 2X
DEAQ 1/2X
DEAQ 1X
DEAQ 2X
Solvent
E
PPQ Survival
Survival Probability
Days
Drug condition
Blood only
IVM 1X
PPQ 1/2X
PPQ 1X
PPQ 2X
Solvent
F
SP Survival
Survival Probability
Days
Drug condition
Blood only
IVM 1x
PYR 1/2X
PYR 2X
SDX 1/2X
SDX 2X
SP 1/2X
SP 2X
Solvent
G
Total Eggs Laid
Blood only
Solvent
AQ 2X
AQ 1X
AQ 1/2X
DEAQ 2X
DEAQ 1X
DEAQ 1/2X
H
PPQ Fertility
Total Eggs Laid
Blood only
Solvent
PPQ 2X
PPQ 1X
PPQ 1/2X
I
SP Fertility
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SP 2X
SP 1/2X

Figure 1

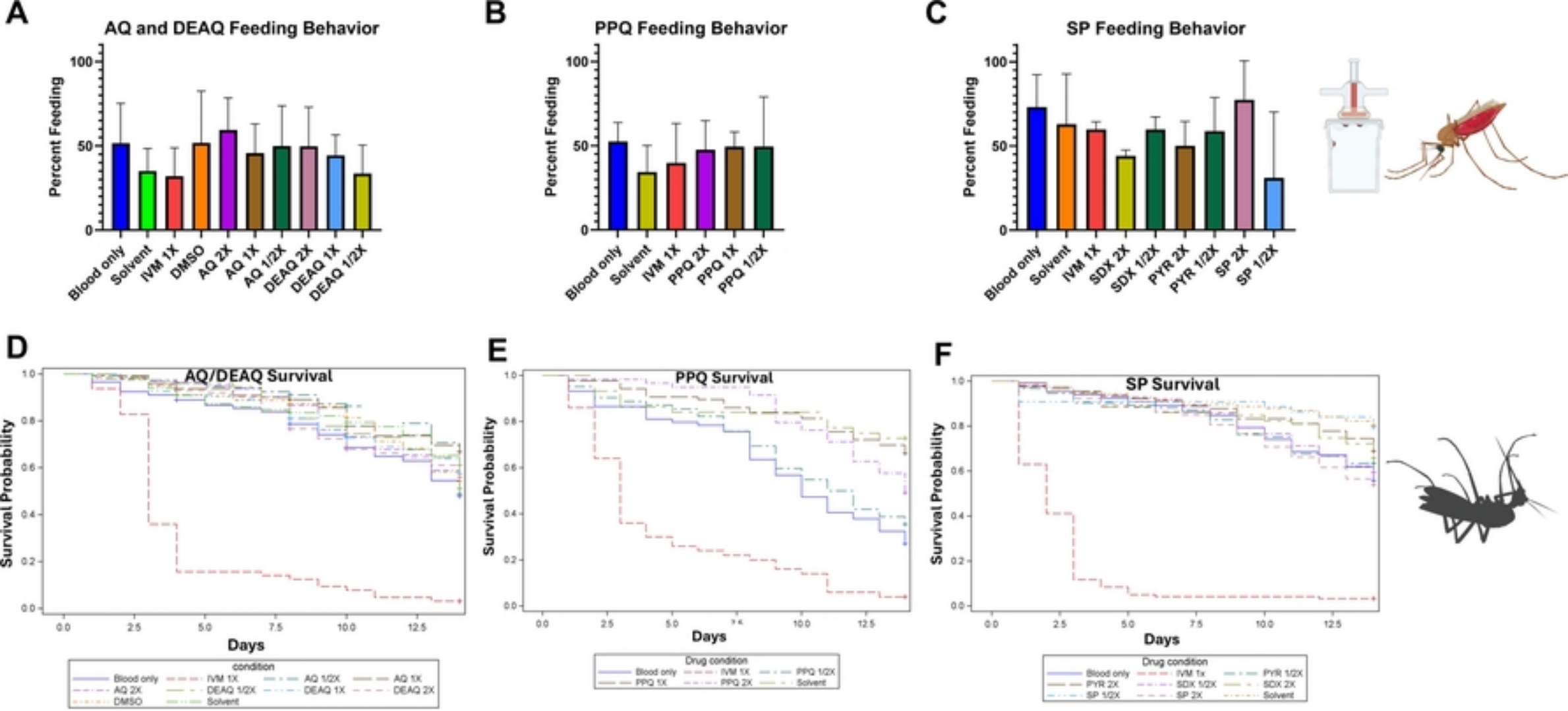
A
AQ and DEAQ Feeding Behavior
Percent Feeding
100
50
0
Blood only
Solvent
IVM 1X
DMSO
AQ 2X
AQ 1X
AQ 1/2X
DEAQ 2X
DEAQ 1X
DEAQ 1/2X
B
PPQ Feeding Behavior
Percent Feeding
100
50
0
Blood only
Solvent
IVM 1X
PPQ 2X
PPQ 1X
PPQ 1/2X
C
SP Feeding Behavior
Percent Feeding
100
50
0
Blood only
Solvent
IVM 1X
SDX 2X
SDX 1/2X
PYR 2X
PYR 1/2X
SP 2X
SP 1/2X
D
AQ/DEAQ Survival
Survival Probability
Days
condition
Blood only
IVM 1X
AQ 1/2X
AQ 1X
AQ 2X
DEAQ 1/2X
DEAQ 1X
DEAQ 2X
DMSO
Solvent
E
PPQ Survival
Survival Probability
Days
Drug condition
Blood only
IVM 1X
PPQ 1/2X
PPQ 1X
PPQ 2X
Solvent
F
SP Survival
Survival Probability
Days
Drug condition
Blood only
IVM 1x
PYR 1/2X
PYR 2X
SDX 1/2X
SDX 2X
SP 1/2X
SP 2X
Solvent

Figure 2

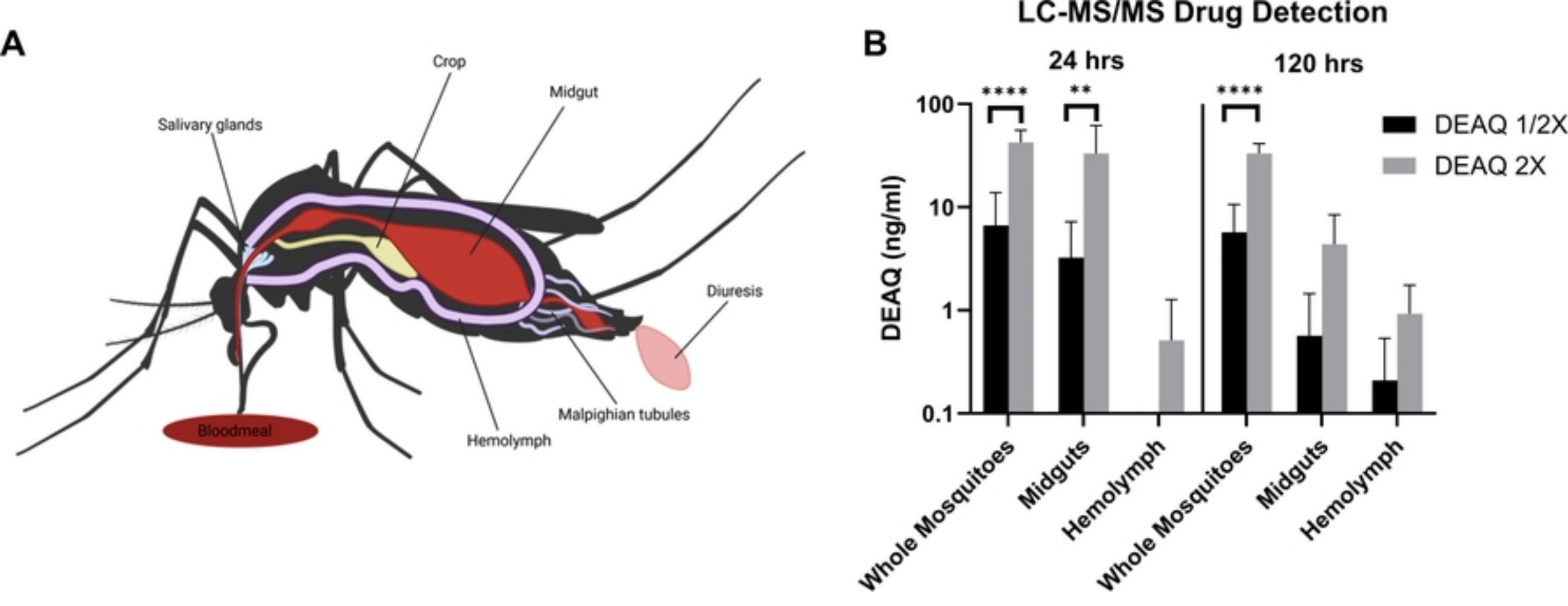
A
Crop
Midgut
Salivary glands
Diuresis
Malpighian tubules
Bloodmeal
Hemolymph
B
LC-MS/MS Drug Detection
24 hrs
120 hrs
****
**
****
100
10
1
0.1
DEAQ (ng/ml)
DEAQ 1/2X
DEAQ 2X
Whole Mosquitoes
Midguts
Hemolymph
Whole Mosquitoes
Midguts
Hemolymph

Figure 3