

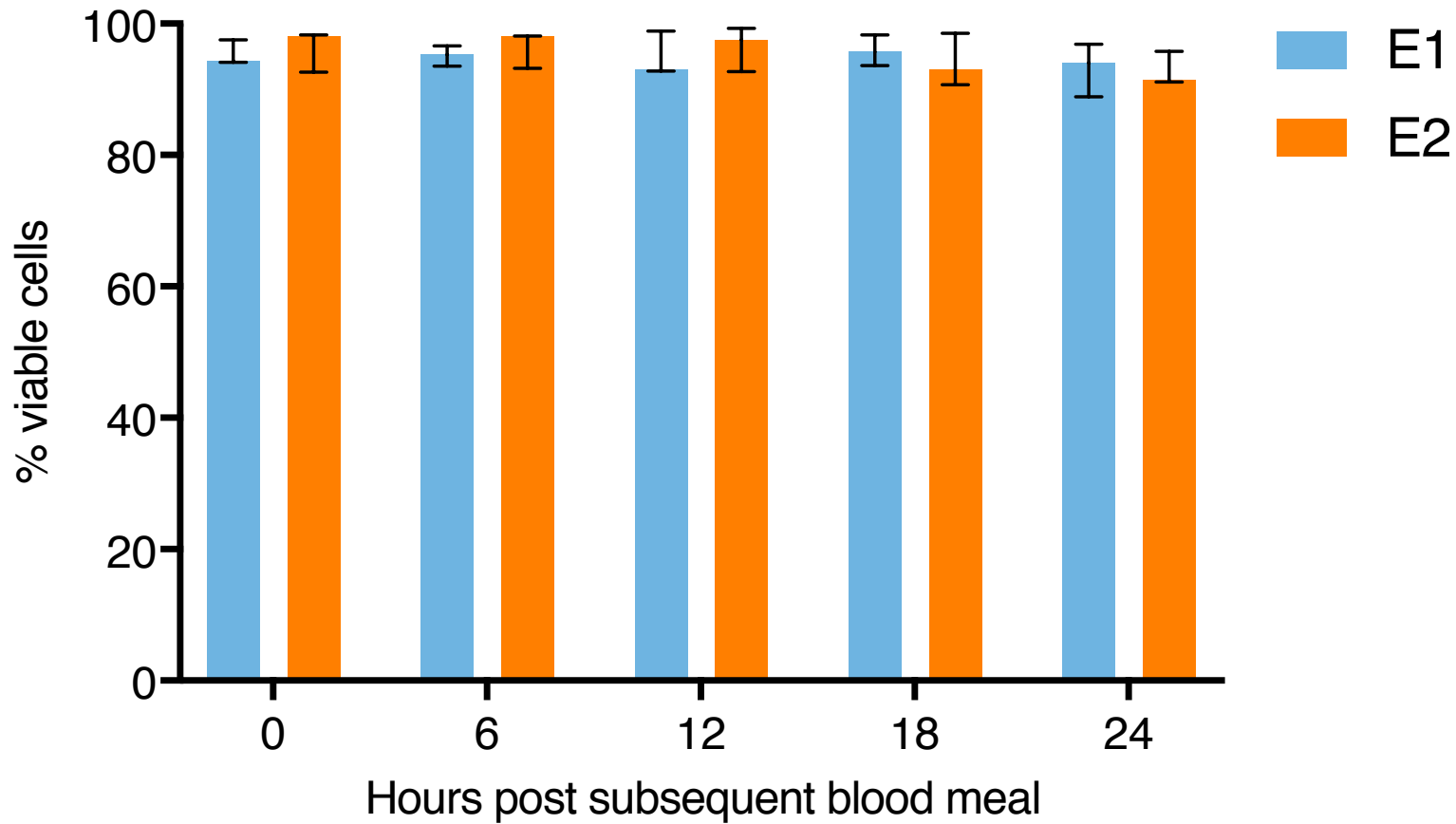
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Supplementary Information

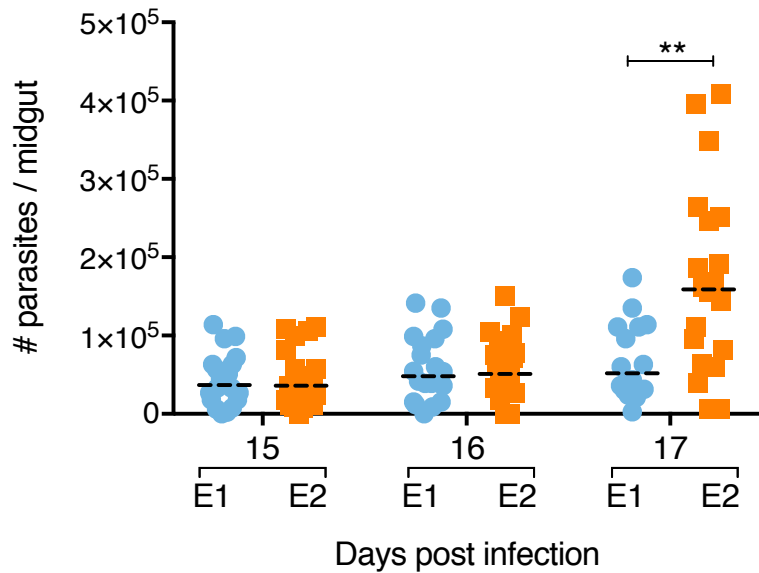
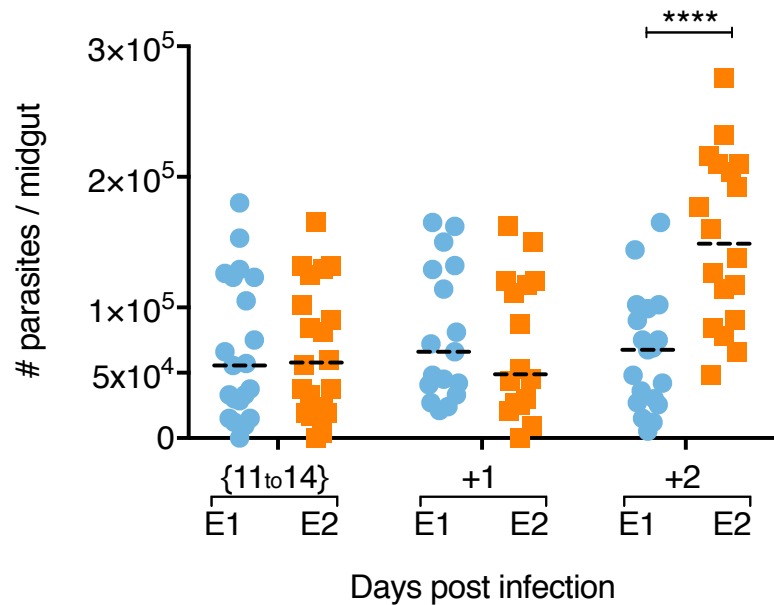
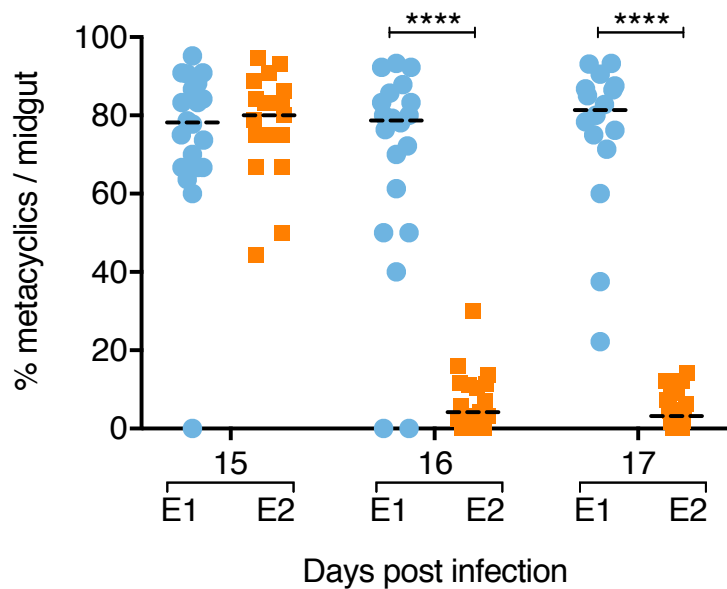
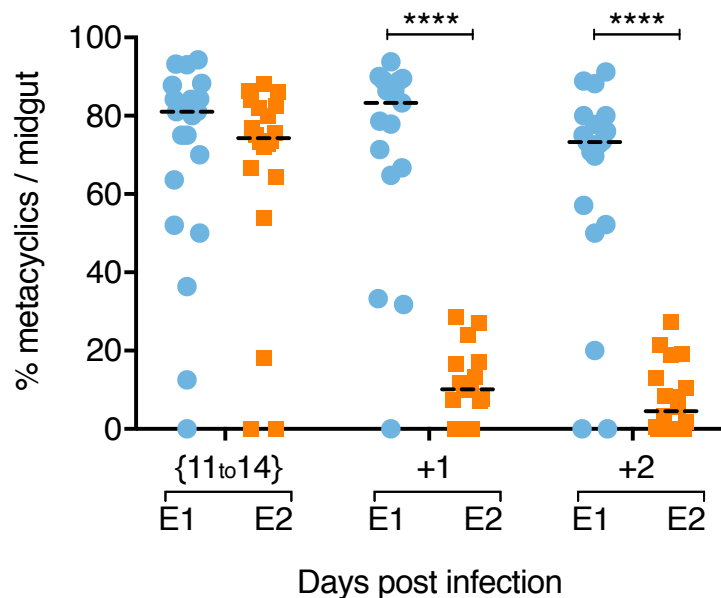
**Sequential blood meals augment vector infectiousness by promoting
Leishmania replication and triggering amplification of metacyclics via
a novel retroleptomonad developmental stage**

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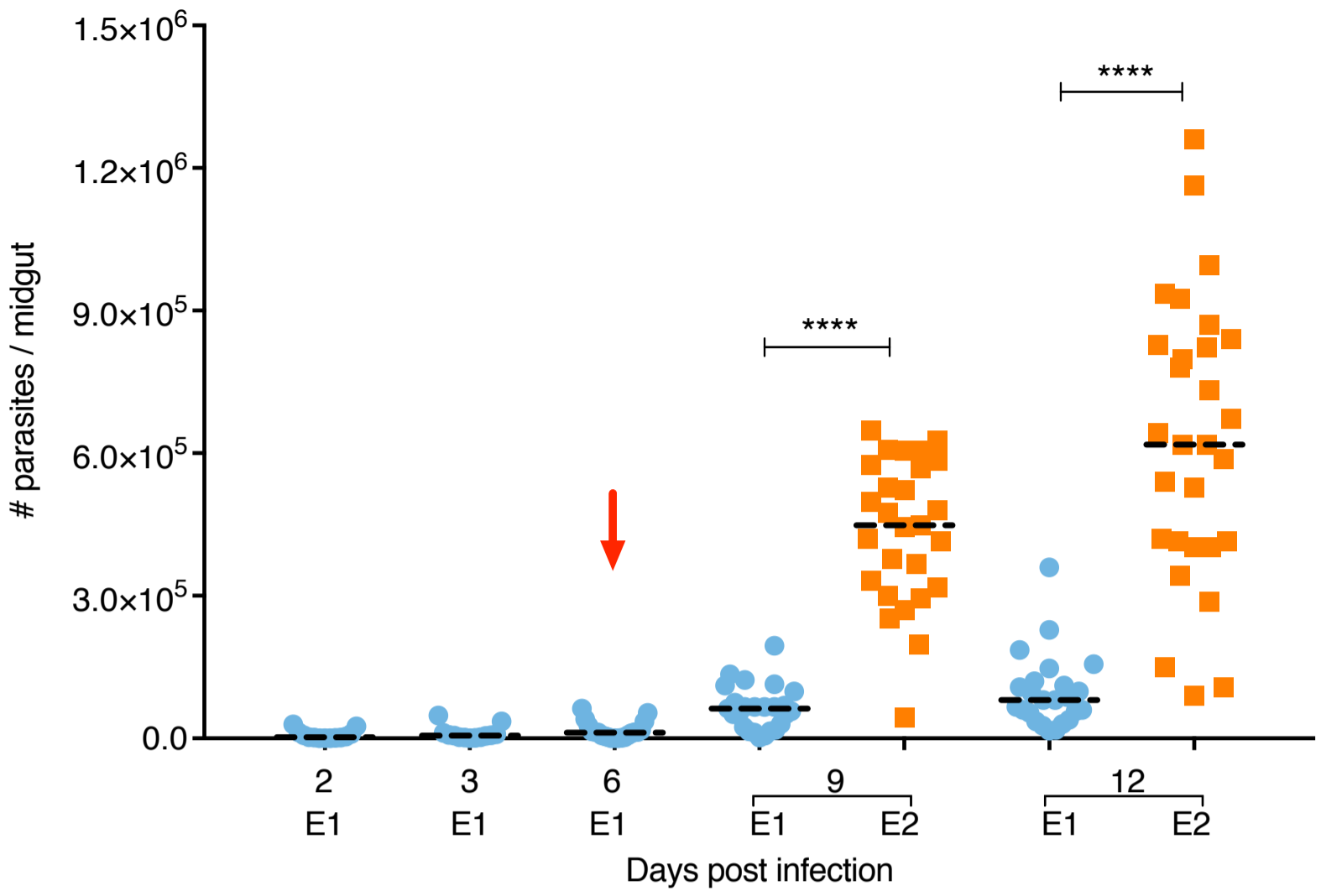
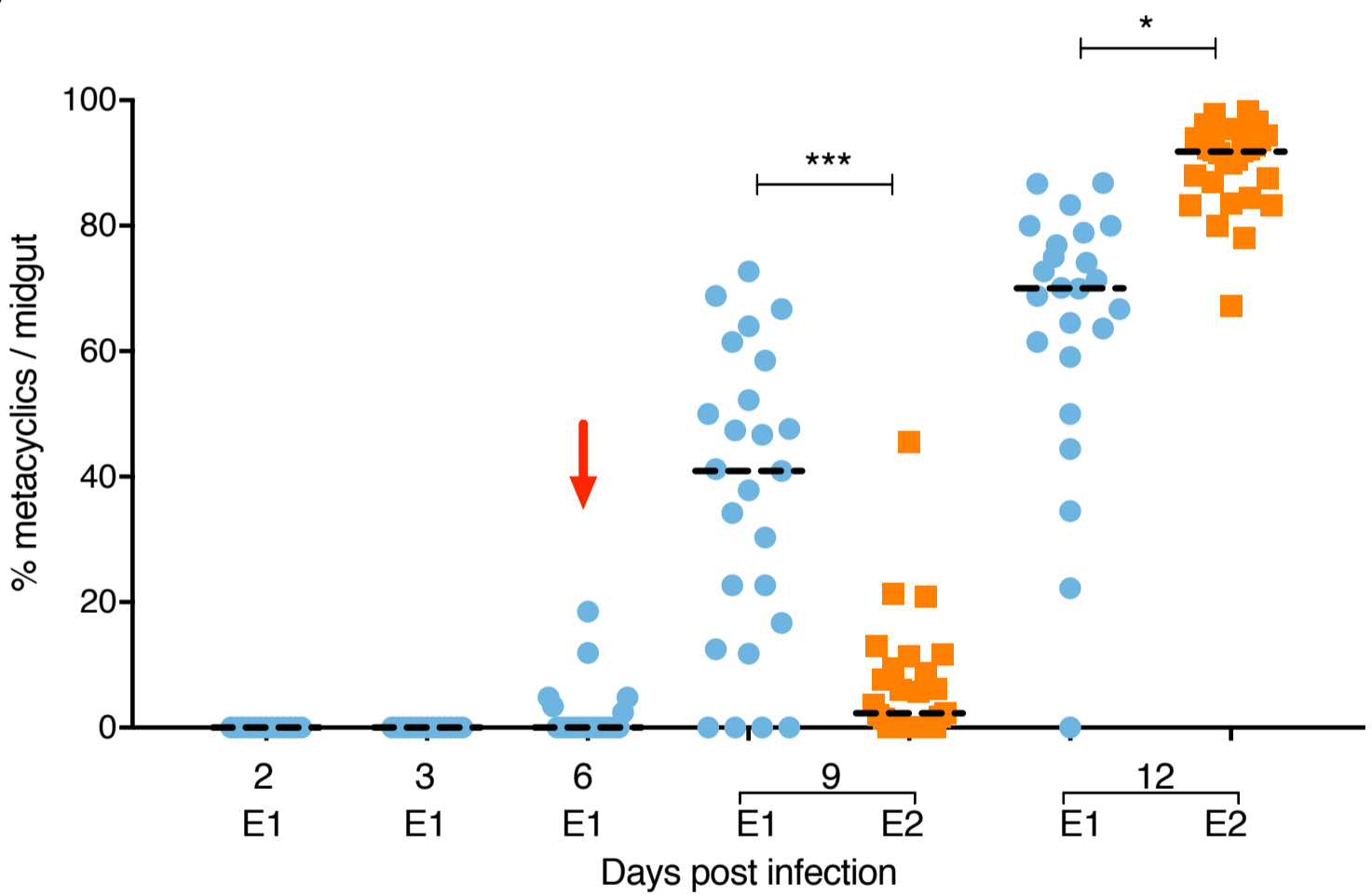
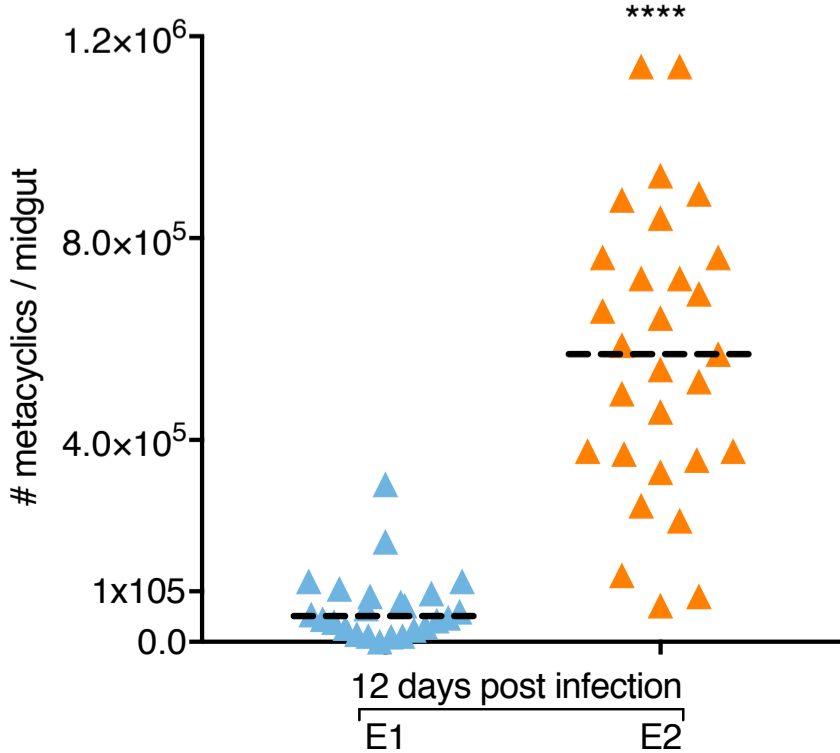
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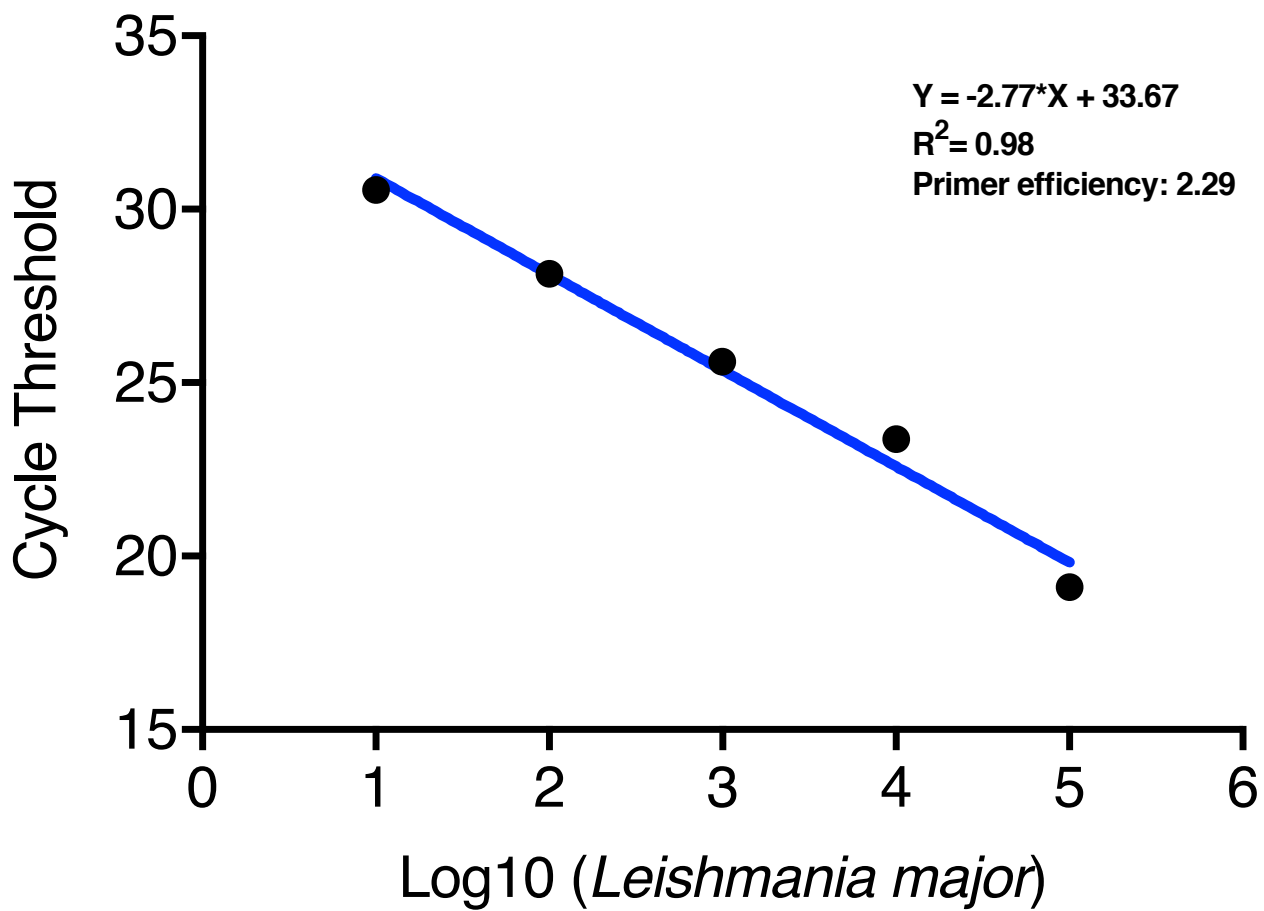
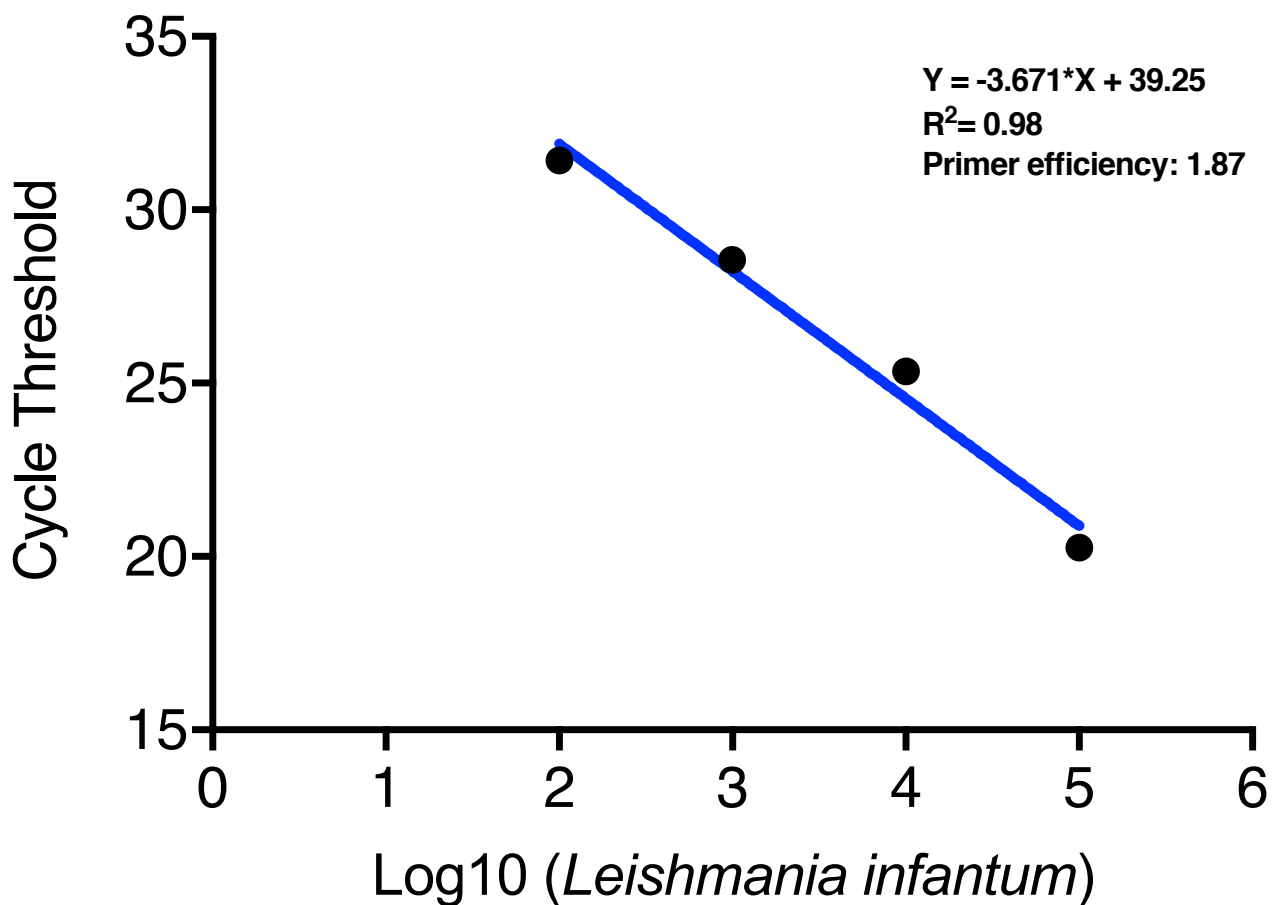
Supplementary Figure 1. Metacyclics remain viable as they transform into retroleptomonads after a subsequent uninfected blood meal. Viability of parasites recovered from E1 or E2 *Leishmania infantum*-infected *Lutzomyia longipalpis* sand flies. was assessed using propidium iodide incorporation followed by flow cytometry. E1, sand flies engorged on an infected blood meal. E2, infected sand flies engorged on a subsequent uninfected blood meal. Median \pm interquartile range are shown. Cumulative data shown from three independent experiments; n=10 sand flies for each independent experiment.

a**b****c****d**

Supplementary Figure 2. Differentiation of *Leishmania* metacyclics into replicative retroleptomonads after a subsequent uninfected blood meal is ubiquitous. (a to d) Midgut parasite number (**a** and **b**) and percent metacyclics (**c** and **d**) in *Leishmania major*-infected *Phlebotomus papatasi* (**a** and **c**) or *L. donovani*-infected *Lu. longipalpis* sand flies (**b** and **d**). E1, sand flies engorged on an infected blood meal. E2, infected sand flies engorged on a subsequent uninfected blood meal. Dashed bar, Median. Cumulative data shown from two independent experiments; n for each condition is specified in Supplementary Table 1. ** $P \leq 0.01$, **** $P \leq 0.0001$ determined by Mann-Whitney's U-test for parasite number and by N1-Chi-squared test for percent metacyclics.

a**b****c**

Supplementary Figure 3. A subsequent avian uninfected blood meal enhances early *Leishmania* infection in the sand fly. (a and b) Midgut parasite number (a), and percent (b) and number (c) of metacyclics in *Leishmania infantum*-infected *Lutzomyia longipalpis* sand flies. E1, sand flies engorged on an infected blood meal. E2, infected sand flies engorged on a subsequent uninfected avian blood meal. Bar, median. Red arrow, timing of subsequent uninfected blood meal. Cumulative data shown from two independent experiments; n for each condition is specified in Supplementary Table 2. * $P < 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$ determined by Mann-Whitney's U-test for parasite number and by N1-Chi-squared test for percent metacyclics.

a**b**

QPCR primers and Probe

Target	Forward primer	Probe	Reverse primer
KDNA minicircle	5'-CCTATTTTACACCAACCCCGT-3'	5 -RAAARKKVRTRCA GAAAYCCCGT-3	5'-GGGTAGGGGCGTTCTGCGAAA-3'

Supplementary Figure 4

***Leishmania* load quantification by qPCR: Standard curve, primers, and probes.**

Standard curve (STD) generated using DNA extracted from single sand flies spiked with a known number of *Leishmania infantum* (**a**) or *L. major* (**b**) parasites. Table provides information on the primers and probe used for parasite amplification. Representative of STDs used to generate data shown in Fig. 3 a and b; n for each condition is specified in Supplementary Table 3.

Supplementary Table 1: Descriptive statistics of sand fly infection experiments for Fig. 1 and Supplementary Figure 2

Related to Fig. 1b,c and f

Day post infection	Day post 2nd blood meal	Group	No. of flies	Total parasite load		Metacyclics load		% of metacyclics (median/mean)
				median (iqr)	mean (\pm sd)	median (iqr)	mean (\pm sd)	
2	-	E1	33	600 (0-1650)	1,236 (\pm 1,688)	0 (0-0)	0 (\pm 0)	0 / 0
3	-	E1	32	6,300 (2,775-9,450)	7,369 (\pm 6,773)	0 (0-0)	0 (\pm 0)	0 / 0
6	-	E1	31	19,200 (6,000-28,200)	20,071 (\pm 17,324)	0 (0-1,200)	1,248 (\pm 2,457)	0 / 4.4
9	-	E1	39	66,000 (33,000-114,000)	78,531 (\pm 58,198)	24,000 (6,000-69,000)	39977 (\pm 40,332)	47.4 / 40.7
12	0	E1	48	96,000 (48,000-137,250)	101,744 (\pm 70,298)	75,750 (28,875-110,250)	81,169 (\pm 61,663)	78.9 / 72.2
		E2	49	111,000 (45,000-162,000)	110,749 (\pm 79,648)	81,000 (31,500-133,500)	90,582 (\pm 73,399)	79 / 72.9
13	1	E1	42	104,250 (63,750-160,125)	116,293 (\pm 85,477)	75,750 (42,000-138,000)	93,429 (\pm 79,999)	77.4 / 76.0
		E2	39	108,000 (66,000-150,000)	109,446 (\pm 56,721)	6,000 (0-16,500)	12,562 (\pm 18,604)	5.6 / 9.0
14	2	E1	42	111,450 (80,625-160,050)	114,250 (\pm 55,767)	90,000 (65,625-132,750)	95,657 (\pm 51,614)	81 / 78.4
		E2	46	226,500 (122,250-319,500)	254,785 (\pm 170,325)	0 (0-6,975)	9,828 (\pm 20,840)	0 / 4.9
15	3	E1	25	126,000 (67,500-178,200)	131,052 (\pm 78,321)	87,000 (45,000-151,500)	102,600 (\pm 72,865)	81.3 / 74.2
		E2	29	288,000 (231,000-387,000)	321,000 (\pm 138,507)	6,000 (0-129,000)	79,138 (\pm 138,628)	0 / 2.6
18	6	E1	21	123,000 (90,000-156,000)	123,471 (\pm 71,126)	105,000 (63,000-123,000)	101,071 (\pm 57,960)	80 / 76.6
		E2	32	519,000 (318,000-682,500)	509,625 (\pm 250,311)	474,000 (303,000-664,500)	480,656 (\pm 240,856)	94.4 / 93.8

Related to Supplementary Figure 2a and c

Day post infection	Day post 2nd blood meal	Group	No. of flies	Total parasite load		Metacyclics load		% of metacyclics (median/mean)
				median (iqr)	mean (\pm sd)	median (iqr)	mean (\pm sd)	
15	0	E1	20	36,750 (18,000-63,000)	43,620 (\pm 33,228)	24,000 (6,375-56,250)	33,675 (\pm 30,745)	78.2 / 74.6
		E2	20	36,000 (12,000-75,000)	44,850 (\pm 37,238)	27,000 (9,000-66,000)	37,650 (\pm 35,073)	80 / 77.6
16	1	E1	20	48,000 (20,250-93,750)	57,930 (\pm 41,646)	36,000 (14,250-73,500)	45,315 (\pm 35,875)	78.7 / 67.8
		E2	19	51,000 (33,300-93,000)	61,753 (\pm 40,288)	3,000 (0-9,000)	6,158 (\pm 10,331)	4.2 / 6.8
17	2	E1	16	51,750 (30,000-111,000)	67,669 (\pm 49,439)	43,500 (19,500-93,750)	57,225 (\pm 46,921)	81.4 / 75.4
		E2	20	159,000 (67,500-250,500)	169,200 (\pm 120,041)	4,500 (0-12,000)	10,200 (\pm 13,431)	3.2 / 4.5

Related to Supplementary Figure 2b and d

Day post infection	Day post 2nd blood meal	Group	No. of flies	Total parasite load		Metacyclics load		% of metacyclics (median/mean)
				median (iqr)	mean (\pm sd)	median (iqr)	mean (\pm sd)	
{11 to 14}	0	E1	21	55,500 (21,750-123,000)	66,957 (\pm 53,737)	45,000 (11,250-102,000)	55271 (\pm 49218)	81 / 69.9
		E2	20	57,750 (20,625-118,875)	67,335 (\pm 50,191)	45,750 (15,750-95,250)	52050 (\pm 42533)	74.3 / 65.6
+1	1	E1	17	66,000 (36,900-130,500)	79,518 (\pm 51,376)	33,000 (23,250-108,000)	60988 (\pm 47511)	83.3 / 71.6
		E2	16	48,750 (25,875-119,250)	70,031 (\pm 52,995)	6,750 (1,500-12,000)	9281 (\pm 10553)	10.2 / 11.9
+2	2	E1	19	67,500 (27,000-99,000)	64,705 (\pm 44,553)	36,000 (21,000-69,000)	46658 (\pm 35203)	73.3 / 63.0
		E2	18	148,950 (88,500-210,000)	152,100 (\pm 65,652)	7,950 (0-18,000)	10433 (\pm 11801)	4.5 / 7.7

(iqr) interquartile range

(sd) standard deviation

Supplementary Table 2: Descriptive statistics of sand fly infection experiments for Fig. 2 and Supplementary Figure 3

Related to Fig. 2a,c and e

Day post infection	Day post 2nd blood meal	Group	No. of flies	Total parasite load		Metacyclics load		% of metacyclics (median/mean)
				median (iqr)	mean (\pm sd)	median (iqr)	mean (\pm sd)	
2	-	E1	37	1,500 (0-2,850)	5,157 (\pm 14,261)	0 (0-0)	0 (\pm 0)	0 / 0
3	-	E1	34	4,800 (2,025-12,225)	6,529 (\pm 5,358)	0 (0-0)	0 (\pm 0)	0 / 0
6	-	E1	43	18,300 (7,500-38,400)	24,719 (\pm 22,171)	0 (0-2,700)	2,051 (\pm 3,887)	0 / 4.9
9	2	E1	38	46,500 (29,625-94,500)	66,797 (\pm 53,131)	25,500 (7,125-60,000)	41,368 (\pm 44,243)	51.8 / 49.6
		E2	44	156,000 (120,000-251,250)	200,659 (\pm 120,612)	6,000 (0-15,000)	13,705 (\pm 23,049)	5.1 / 7.4
12	6	E1	33	96,000 (26,250-160,500)	103,645 (\pm 79,080)	84,000 (14,550-139,500)	85,291 (\pm 69,052)	77.1 / 73.1
		E2	47	438,000 (246,000-558,000)	404,872 (\pm 188,038)	408,000 (210,000-498,000)	366,638 (\pm 179,105)	90.9 / 89.1

Related to Fig. 2b,d and f

Day post infection	Day post 2nd blood meal	Group	No. of flies	Total parasite load		Metacyclics load		% of metacyclics (median/mean)
				median (iqr)	mean (\pm sd)	median (iqr)	mean (\pm sd)	
2	-	E1	10	13,800 (5,625-25,050)	15,360 (\pm 10,668)	0 (0-0)	0 (\pm 0)	0 / 0
3	-	E1	13	18,000 (9,750-26,100)	20,238 (\pm 13,972)	0 (0-0)	0 (\pm 0)	0 / 0
6	-	E1	20	1,650 (375-8,250)	4,350 (\pm 4,940)	0 (0-0)	285 (\pm 834.3)	0 / 4.7
9	2	E1	18	6,000 (3,600-25,650)	13,317 (\pm 13,948)	1,800 (0-15,300)	6,683 (\pm 8,426)	31.4 / 32.8
		E2	19	222,000 (60,000-264,000)	199,342 (\pm 146,565)	15,000 (6,000-108,000)	58,058 (\pm 69,568)	19.4 / 21.6
12	6	E1	22	3,300 (0-16,200)	10,364 (\pm 14,207)	1,500 (0-9,750)	7,391 (\pm 12,011)	28.2 / 34.9
		E2	22	118,500 (55,500-190,500)	162,314 (\pm 159,932)	97,500 (43,500-168,000)	142,173 (\pm 145,345)	85.2 / 84.4

Related to Supplementary Figure 3a,b and c

Day post infection	Day post 2nd blood meal	Group	No. of flies	Total parasite load		Metacyclics load		% of metacyclics (median/mean)
				median (iqr)	mean (\pm sd)	median (iqr)	mean (\pm sd)	
2	-	E1	13	2400 (900-11,100)	7,338 (\pm 9,669)	0 (0-0)	0 (\pm 0)	0 / 0
3	-	E1	13	6,000 (2,100-10,050)	10,638 (\pm 14,488)	0 (0-0)	0 (\pm 0)	0 / 0
6	-	E1	21	12,300 (2,400-22,200)	16,143 (\pm 18,027)	0 (0-300)	671.4 (\pm 1,749)	0 / 2.2
9	2	E1	25	63,000 (21,000-87,000)	63,120 (\pm 46,550)	21,000 (3,000-45,000)	28,560 (\pm 29,232)	40.9 / 36.3
		E2	27	448,500 (318,000-576,000)	437,078 (\pm 150,921)	14,400 (0-43,200)	28,989 (\pm 37,321)	2.3 / 6.6
12	6	E1	24	81,000 (42,750-117,825)	96,825 (\pm 77,473)	51,000 (24,750-94,500)	69,813 (\pm 68,936)	70.05 / 64.2
		E2	29	618,000 (408,000-833,850)	627,093 (\pm 298,379)	570,000 (366,000-762,000)	570,683 (\pm 286,144)	91.8 / 89.7

(iqr) interquartile range

(sd) standard deviation

Supplementary Table 3: Descriptive statistics of sand fly infection experiments for Fig. 3

Related to Fig. 3a and b

Day post infection	Group	No. of flies	No. of amastigotes	
			median (iqr)	mean (\pm sd)
0	L. longipalpis / L. infantum	57	52 (11.5-178.5)	586 (\pm 2,535)
	P. papatasi / L. major	35	80 (5-941)	835.9 (\pm 2,144)

Related to Fig. 3c,e and g

Day post infection	Day post 2nd blood meal	Group	No. of flies	Total parasite load		Metacyclics load		% of metacyclics (median/mean)
				median (iqr)	mean (\pm sd)	median (iqr)	mean (\pm sd)	
2	-	E1	26	0 (0-600)	323.1 (\pm 440.3)	0 (0-0)	0 (\pm 0)	0 / 0
3	-	E1	26	0 (0-375)	473.1 (\pm 1022)	0 (0-0)	0 (\pm 0)	0 / 0
6	-	E1	30	150 (0-3,600)	3,840 (\pm 8,689)	0 (0-0)	0 (\pm 0)	0 / 0
9	3	E1	41	0 (0-5,550)	8,334 (\pm 18,941)	0 (0-0)	2,751 (\pm 7,331)	0 / 7.9
		E2	41	69,000 (2,250-234,000)	132,307 (\pm 166,307)	0 (0-0)	439 (\pm 2,811)	0 / 0.1
12	6	E1	45	1200 (0-12,900)	8,380 (\pm 12,951)	0 (0-600)	2,080 (\pm 5,967)	0 / 7.2
		E2	46	82,500 (0-227,250)	131,739 (\pm 158,280)	58,500 (0-180,750)	107,876 (\pm 140,472)	68.3 / 51.3

Related to Fig. 3d,f and h

Day post infection	Day post 2nd blood meal	Group	No. of flies	Total parasite load		Metacyclics load		% of metacyclics (median/mean)
				median (iqr)	mean (\pm sd)	median (iqr)	mean (\pm sd)	
2	-	E1	26	2,100 (600-10,050)	13,546 (\pm 24,567)	0 (0-0)	0 (\pm 0)	0 / 0
3	-	E1	31	300 (0-2,700)	3,929 (\pm 7,945)	0 (0-0)	0 (\pm 0)	0 / 0
6	-	E1	35	600 (0-3,000)	2,040 (\pm 3,191)	0 (0-0)	8.6 (\pm 51)	0 / 0.2
9	3	E1	39	300 (0-6,600)	6,346 (\pm 13,651)	0 (0-900)	3,277 (\pm 9,245)	0 / 13.6
		E2	36	79,500 (4,200-173,250)	114,608 (\pm 142,744)	0 (0-6,000)	7,667 (\pm 17,849)	0 / 3.6
12	6	E1	43	600 (0-13,800)	11,463 (\pm 21,838)	0 (0-9,000)	5,763 (\pm 11,691)	0 / 22.8
		E2	43	75,000 (900-249,000)	133,263 (\pm 153,919)	60,000 (0-222,000)	117,614 (\pm 139,703)	86 / 63.4

(iqr) interquartile range

(sd) standard deviation

2. Supplementary Videos

Supplementary Video 1. Fast swimming metacyclics at mature infection. Thoracic midgut of an infected E1 sand fly releasing fast swimming, small-bodied, metacyclics with long flagella.

Leishmania infantum-infected *Lutzomyia longipalpis* sand flies (E1) fourteen days post infection, the thoracic midgut of E1 sand flies was separated for imaging immediately after dissection.

Scale, 20 μm .

Supplementary Video 2. Fast swimming metacyclics at mature infection. Thoracic midgut of an infected E1 sand fly releasing fast swimming, small-bodied, metacyclics with long flagella.

Dispersed parasites (from Supplementary Video 1) highlight shape and speed of metacyclics.

Leishmania infantum-infected *Lutzomyia longipalpis* sand flies (E1) fourteen days post infection, the thoracic midgut of E1 sand flies was separated for imaging immediately after dissection.

Scale, 20 μm .

Supplementary Video 3. Fast swimming metacyclics dedifferentiate into slow moving retroleptomonads after a subsequent uninfected blood meal. Thoracic midgut of infected E2 sand fly releasing slow moving large retroleptomonds with short flagella. *Leishmania infantum*-infected *Lutzomyia longipalpis* sand flies (E1) were allowed to take a second blood meal on an uninfected mouse 12 days post infection (E2). Fourteen days post infection and 2 days post

second blood meal, the thoracic midgut of E2 sand flies was separated for imaging immediately after dissection. Scale, 20 μm .

Supplementary Video 4. Fast swimming metacyclics dedifferentiate into slow moving retroleptomonads after a subsequent uninfected blood meal. Thoracic midgut of infected E2 sand fly releasing slow moving large retroleptomonds with short flagella. *Leishmania infantum*-infected *Lutzomyia longipalpis* sand flies (E1) were allowed to take a second blood meal on an uninfected mouse 12 days post infection (E2). Dispersed parasites (from Supplementary Video 3) highlight shape and speed of retroleptomonads. Fourteen days post infection and 2 days post second blood meal, the thoracic midgut of E2 sand flies was separated for imaging immediately after dissection. Scale, 20 μm .

Supplementary Video 5. Dedifferentiation of a metacyclic promastigote. *Leishmania major* metacyclic promastigotes were obtained from stationary phase cultures by two rounds of purification by PNA. Parasites attached to the glass base of a dish were imaged every minute for 18h. Arrow indicates representative metacyclic promastigotes undergoing dedifferentiation. The accelerated event shows the diminishing flagellum in relation to an increase in cell body size culminating with an emergent retroleptomonad promastigote that starts to divide.

Supplementary Video 6. Dedifferentiation of a metacyclic promastigote. *Leishmania major* metacyclic promastigotes were obtained from stationary phase cultures by two rounds of purification by PNA. Parasites attached to the glass base of a dish were imaged every minute for

18h. Arrow indicates representative metacyclic promastigotes undergoing dedifferentiation. The accelerated event shows the diminishing flagellum in relation to an increase in cell body size culminating with an emergent retroleptomonad promastigote that starts to divide.

Supplementary Video 7. Dedifferentiation of a metacyclic promastigote. *Leishmania major* metacyclic promastigotes were obtained from stationary phase cultures by two rounds of purification by PNA. Parasites attached to the glass base of a dish were imaged every minute for 18h. Arrow indicates representative metacyclic promastigotes undergoing dedifferentiation. The accelerated event shows the diminishing flagellum in relation to an increase in cell body size culminating with an emergent retroleptomonad promastigote that starts to divide.

Supplementary Video 8. The haptomonads parasite sphere of an infected sand fly after a subsequent uninfected blood meal. *Leishmania infantum*-infected *Lutzomyia longipalpis* sand flies were allowed to take a second blood meal on an uninfected mouse 12 days post infection. Fifteen days post infection and three days post second blood meal, haptomonads spheres were separated for imaging immediately after dissection. The haptomonads parasite spheres were separated by pulling the crop and the midgut apart, which resulted in the removal of intact structures from the cardiac valve. A representative haptomonad parasite sphere of flies from two independent experiments is shown in the video.