Stumpy forms are the predominant transmissible forms of *Trypanosoma brucei*.

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Abstract

Schuster *et al.* demonstrated that bloodstream slender forms of African trypanosomes are readily transmissible to young tsetse flies where they can complete their complex life cycle (1). In their experimental conditions, a single slender parasite was sufficient for productive infection. Here, we compared the infectivity of slender and stumpy bloodstream forms in adult flies with a mature immune system, and without using any chemical compounds that would alter the insect immune response and/or promote the infection. After ingestion of slender forms, infected flies were observed only in one out of 24 batches of non-immunocompetent teneral flies and with a high number of parasites. In contrast, infected flies were detected in 75% (18/24) of the batches infected with stumpy parasites, and as few as 10 stumpy parasites produced mature infections in immune adult flies. We discuss that, although Schuster *et al.* have demonstrated the intrinsic capacity of slender form trypanosomes to infect young and naive tsetse flies, highlighting the remarkable plasticity and adaptability of these protists, this phenomenon is unlikely to significantly contribute to the epidemiology of African trypanosomiases. According to both experimental and field observations, stumpy forms appear to be the most adapted forms for African trypanosome transmission from the mammalian host to the tsetse fly vector in natural conditions.

Introduction

Protist parasites of the *Trypanosoma brucei* group cause Human African Trypanosomiasis (HAT), or sleeping sickness in humans, and nagana in cattle (2). They are transmitted by the blood feeding tsetse fly following a long (at least 3 weeks) and complex (at least 9 distinct stages) cyclical development (review in (3)). In the mammalian host's blood circulation, proliferating slender trypanosomes differentiate into cell cycle-arrested stumpy cells upon quorum sensing when they reach high parasite densities (4-7). This differentiation is thought not only to regulate the parasite load in the reservoir host (8), but also to provide transmissible parasites adapted to pursue the life cycle in the vector host (9). Indeed, stumpy forms express several transcripts and proteins necessary to the next developmental stage in the insect, the procyclic form, including the Protein Associated to Differentiation 1 or PAD1 (5). For decades, arrest of the cell cycle and differentiation to the stumpy stage were presumed essential for the developmental progression of bloodstream trypanosomes to the insect stages.

Recently, Schuster *et al.* demonstrated that slender trypanosomes can also present some intrinsic characteristic of transmissible forms (PAD1 mRNAs and proteins) and are readily transmissible to both young male and female tsetse flies, where they can complete their complex life cycle (1), yet with a lower efficiency than stumpy forms (10). In their experimental conditions, a single slender parasite was sufficient for productive infection. These laboratory conditions are however significantly different from what is encountered in the field. First, only young teneral flies (1-3 days post-eclosion) with an immature immune system were used. Second, in some experiments, chemical compounds altering the insect immune response (glutathione) and/or promoting the infection (N-acetyl-glucosamine) were added to the infective meal. To assess the importance of these parameters, we challenged the infectivity of slender bloodstream forms in adult tsetse flies, i.e. in conditions closer to the natural situation.

Material and methods

Strains, culture and *in vitro* differentiation

The AnTat 1.1E Paris pleomorphic strain of *Trypanosoma brucei brucei* was derived from a strain originally isolated from a bushbuck in Uganda in 1966 (11). Bloodstream form trypanosomes were cultivated in HMI-9 medium supplemented with 10% (v/v) FBS (12) at 37°C in 5% CO_2. Proliferative slender cells were maintained at densities

65 lower than 5.10^5 parasites/ml to prevent their natural quorum-sensing-dependent
66 differentiation into stumpy forms. For *in vitro* slender to stumpy BSF differentiation, we
67 used 8-pCPT-2′-O-Me-5′-AMP, a nucleotide analogue of 5'-AMP (BIOLOG Life
68 Science Institute, Germany). Briefly, $2x10^6$ pleomorphic AnTat 1.1E slender forms
69 were incubated with 8-pCPT-2′-O-Me-5′-AMP (5 µM) for 48 h (13). Freshly
70 differentiated stumpy forms and slender cells were then centrifuged at 1,400 × g for 10
71 minutes and resuspended at the appropriate densities in SDM-79 medium
72 supplemented with 10% FBS. Cells were resuspended at either 10^3, 10^4 or 10^5
73 parasites / ml. Assuming individual bloodmeal volumes ranging between 10-100 µl,
74 this would correspond to ingestions of 10-100, 100-1,000 or 1,000-10,000 parasites
75 per condition.

76 **Tsetse fly maintenance, infection and dissection**

77 *Glossina morsitans morsitans* tsetse flies were maintained in Roubaud cages at 27°C
78 and 70% hygrometry and fed through a silicone membrane with fresh mechanically
79 defibrinated sheep blood (BCL, France). Adult (between 2 and 3 weeks after
80 emergence) or teneral males (between 24h and 72h post-emergence) were allowed to
81 ingest parasites through a silicone membrane. No chemical supplement was used in
82 the first set of experiments. For assessing the effect of immunomodulatory compounds
83 in the second set of experiments, 60mM *N*-acetylglucosamine were added to the
84 infective meal. A total of 3 to 5 independent biological replicates per condition were
85 performed with batches of 50 flies per condition.
86 Flies were starved for at least 24 hours before being dissected blindly 28 to 31 days
87 post-ingestion for isolation of all stages from the midgut and salivary glands. For
88 recovery of all tsetse organs, after rapid isolation of the salivary glands in a first drop
89 of phosphate buffer saline (PBS), whole tsetse alimentary tracts, from the distal part of
90 the foregut to the Malpighian tubules, were dissected and arranged lengthways in
91 another drop of PBS as previously described (14, 15). Isolated organs were then
92 scrutinized under a microscope at 40x magnification by two independent readers and
93 infection rates per organ were scored (16).

94 **Immuno-fluorescence analysis (IFA)**

95 Cultured parasites were washed in TDB and spread onto poly-L-lysine coated slides.
96 For flash methanol fixation, slides were air-dried for 10 min, fixed in methanol at -20°C
97 for 5 seconds and rehydrated for 20 min in PBS. For immunodetection of stumpy forms,

98 slides were incubated for 1 h at 37°C with a rabbit polyclonal anti-PAD1 antibody
99 (kindly provided by Keith Matthews, University of Edinburgh) (5) diluted at 1:300 in
100 PBS containing 0.1% Bovine Serum Albumin (BSA). After 3 consecutive 5 min washes
101 in PBS, a species and subclass-specific secondary antibody coupled to the Alexa 488
102 fluorochrome (Jackson ImmunoResearch) diluted at 1:1000 in PBS containing 0.1%
103 BSA was applied for 1 h at 37°C. After washing in PBS, slides were finally stained with
104 4',6-diamidino-2-phenylindole (DAPI, 1 µg/ml) for visualization of kinetoplast and
105 nuclear DNA content and mounted under coverslips with ProLong antifade reagent
106 (Invitrogen), as previously described (14). Slides were observed under an
107 epifluorescence DMI4000 microscope (Leica) with a 100x oil objective (NA 1.4) to
108 assess the proportion of PAD1-positive cells in the infective meals (n > 100 cells /
109 condition).

110 **Statistical analysis**

111 Infections rates were compared by a two-sided ANOVA at 95% confidence with Prism
112 V10.0.3 (GraphPad). MG infection rate comparisons were statistically significant
113 between teneral and adult flies infected with ST in each amount ($p<0.02$ with 10
114 parasites; $p<0.0001$ with 100 and 1,000 parasites) and with 1,000 SL ($p<0.0001$). MG
115 infection rate comparisons were statistically significant ($p<0.0001$) between parasite
116 stages (SL and ST) in each amount (10, 100 and 1,000) and for each fly group (teneral
117 and adult), excepted in teneral flies infected with 1,000 parasites ($p=0.2356$).

118 **Results**

119 Pleomorphic *T. b. brucei* bloodstream forms were either maintained in culture at a
120 density lower than 5.10^5 parasites / ml to prevent quorum-sensing-induced
121 differentiation and obtain only slender forms or induced for differentiation with a 5'-
122 AMP nucleotide analogue to obtain mostly stumpy forms. The expression of PAD1 at
123 the cell surface was assessed by immunofluorescence analysis prior to each
124 experimental infection: no PAD1 expression was detected in the slender group,
125 whereas an average of 63% (52% to 71%, n = 12 replicates) of the induced cells were
126 expressing PAD1. Batches of 50 teneral (< 72h) or adult (2-3 weeks) male tsetse flies
127 were fed in parallel with either slender or stumpy forms at densities corresponding to
128 individual ingestion of about 10, 100 or 1,000 parasites per bloodmeal. In total, 1,384
129 flies from 12 distinct experimental infections were dissected about 4 weeks (28 to 31

130 days) after parasite ingestion. Infection rates in midguts and salivary glands were
131 quantified and plotted for each condition (Figure 1 and Table S1).
132 After ingestion of slender forms, infected flies were observed in only 1 batch out of 24.
133 This occurred in not yet fully immunocompetent teneral flies and with the highest
134 number of ingested parasites (1,000 to 10,000 parasites). In contrast, midgut and
135 salivary glands infected flies were observed in 75% (18/24) and 62.5% (15/24) of the
136 batches infected with stumpy parasites, respectively. As few as 10 stumpy parasites
137 produced mature infections in immunocompetent adult flies and the infection rates
138 were similar whatever the amounts of stumpy forms ingested. In more susceptible non-
139 immune teneral flies, the infection rates were increasing with the number of stumpy
140 forms ingested.
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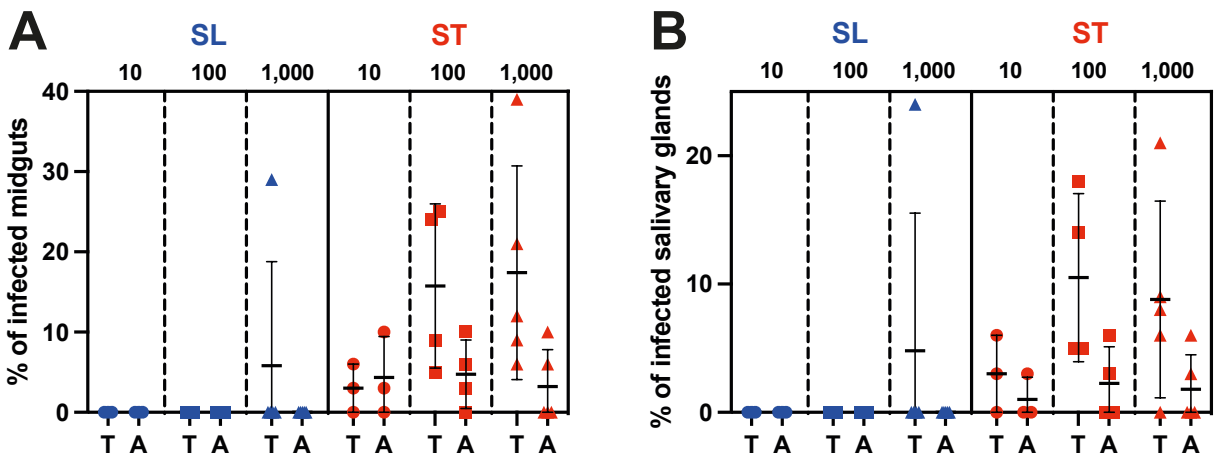

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144 **Figure 1. Stumpy forms are the predominant transmissible forms.** Comparison of
145 (**A**) midgut and (**B**) salivary gland infection rates in teneral (T) Vs. adult (A) tsetse flies
146 (batches of 50 flies) infected with 10 to 100 (circles, 3 independent experiments), 100
147 to 1,000 (squares, 4 independent experiments) or 1,000 to 10,000 (triangles, 5
148 independent experiments) parasites in the slender (SL in blue) or stumpy (ST in red)
149 forms.
150
151 Differences between the strain clones, the cell culture conditions and/or the fly colony
152 maintenance conditions could explain part of the differences in infection rates observed
153 here as compared to the Schuster *et al.* study (1). Nevertheless, the use of the lectin-
154 inhibitory sugar N-acetyl-glucosamine to enhance infection rates in the latter study
155 could be a more likely explanation. To assess this hypothesis, an additional

156 experimental challenge was performed to compare infection rates in teneral versus
157 adult flies, with or without N-acetyl-glucosamine supplement in an infective meal
158 containing 10^5 slender parasites / ml (Figure 2). Whereas no infection was detected in
159 adult flies, the N-acetyl-glucosamine supplementation of the infective meal led to an
160 increase of the infection rates from 2,4% to 13,3% in teneral flies (Figure 2).
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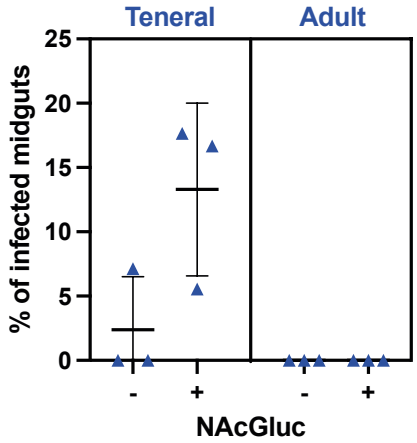

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163 **Figure 2. *N*-acetylglucosamine promotes trypanosome infection in teneral flies.**
164 Comparison of midgut infection rates in teneral Vs. adult tsetse flies (batches of 50
165 flies) infected with 1,000 to 10,000 slender parasites with (+) or without (-) N-acetyl-
166 glucosamine (NAcGluc) supplement in the infective meal containing 10^5 slender
167 parasites / ml (equivalent to 1,000 to 10,000 slender parasites per meal).
168

169 **Discussion**

170 The findings of Schuster *et al.* (1) have opened a debate on the traditional view of the
171 trypanosome life cycle where slender trypanosomes are considered as non-competent
172 for cyclical development in the insect vector (18). The authors proposed that their
173 observations could provide a solution to a long-lasting paradox, namely the successful
174 transmission of parasites in chronic infections, despite low parasitemia. Nonetheless,
175 Schuster *et al.* performed all their experimental infections in laboratory conditions that
176 were optimized for maximum transmission efficiency, with the use of teneral flies, and
177 for some experiments, the addition of *N*-acetylglucosamine or glutathione (1).
178 Teneral flies are young flies that remain unfed for up to 3-5 days after emergence from
179 their puparium. They are known to be significantly more susceptible to trypanosome
180 infection as multiple studies demonstrated they are immunologically immature (weak

181 immune system and, leaky peritrophic matrix) (19-22). According to capture-recapture
182 studies (23), teneral flies however represent a minority of individuals in wild tsetse
183 populations. Hence, knowing that adults can live up to 9 months (24), the impact of
184 teneral flies on trypanosome transmission may be limited, if not incidental.
185 In addition, Schuster *et al.* supplemented most infective meals with 60 mM *N*-
186 acetylglucosamine, an inhibitor of tsetse midgut lectins (17) that was also confirmed to
187 enhance trypanosome infectivity in teneral flies in the present study. For infections with
188 monomorphic parasites, the addition of 12.5 mM glutathione, an antioxidant that
189 reduces the midgut environment, protected trypanosomes from cell death induced by
190 reactive oxygen species (25). In total, these two chemical compounds used in Schuster
191 *et al.* (1) have inhibited the immune response in teneral flies and substantially
192 enhanced the chances for slender trypanosomes to develop in the insect vector.
193 Conditions are far less favourable in the field; hence we investigated and compared
194 the infection potential of slender and stumpy forms in adult and teneral flies without the
195 addition of chemicals. We observed that slender forms were not infective to adult tsetse
196 flies and only at densities higher than 10^5 parasites / ml. Nevertheless, in endemic
197 areas, especially in Western Africa, parasitemia in confirmed cases are usually very
198 low (< 10^4 parasites / ml in Guinea for instance) and it is necessary to concentrate
199 parasites in blood prior to microscopic examination to increase sensitivity of
200 parasitological diagnosis (2). Hence, the possible transmission of a few slender
201 trypanosomes from the blood of individuals with a chronic infection is unlikely to explain
202 the maintenance of the parasite circulation in tsetse populations.
203 By contrast, we observed that as few as 10 stumpy parasites are enough to produce
204 mature infections in both teneral and adult flies, already with a significant efficiency. In
205 patients with low parasitemia, the quorum-sensing-triggered differentiation of slender
206 to stumpy forms could be compatible with, or even more adapted to, extravascular
207 forms present in some tissues and organs, with a limited dilution of the parasites and
208 parasite factors remaining concentrated locally. Indeed, extravascular PAD1-positive
209 trypanosomes were detected in high numbers at least in adipose tissues (26) and in
210 the dermis (27) of experimentally infected mice. The presence of PAD1-positive
211 extravascular trypanosomes was also assessed in the skin of confirmed gambiense
212 HAT cases and unconfirmed seropositive individuals in endemic areas (28) (and
213 unpublished data). This suggests that stumpy trypanosomes accessible to tsetse flies
214 are likely more abundant than previously estimated in individuals with low parasitemia.

Schuster *et al.* have demonstrated the intrinsic capacity of slender form trypanosomes to infect young and naive tsetse flies, highlighting the remarkable plasticity and adaptability of these protists. The fine understanding of the underlying cellular mechanisms and / or transient adaptations involved in this process remains an exciting challenge. This event is however unlikely to contribute to the epidemiology of African trypanosomiases in natural settings. According to both experimental and field observations, stumpy forms appear to be the most adapted forms for African trypanosome transmission from the mammalian host to the tsetse fly vector in natural conditions.

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Author contributions

AC, NP, JMTN and PS performed the experiments. BR designed the study, analysed the data, and wrote the manuscript. JMTN, PS and BR discussed the manuscript.

Competing interest

All authors declare no financial relationships with any organizations that might have an interest in the submitted work in the previous three years, no other relationships or activities that could appear to have influenced the submitted work, and no other relationships or activities that could appear to have influenced the submitted work.

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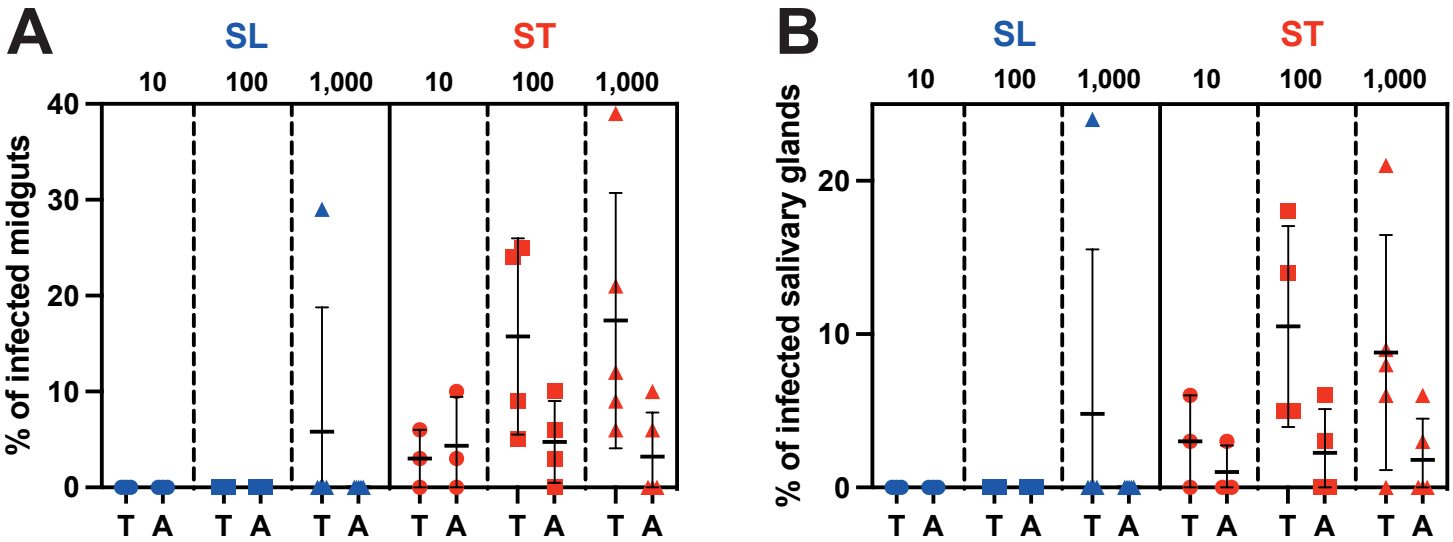
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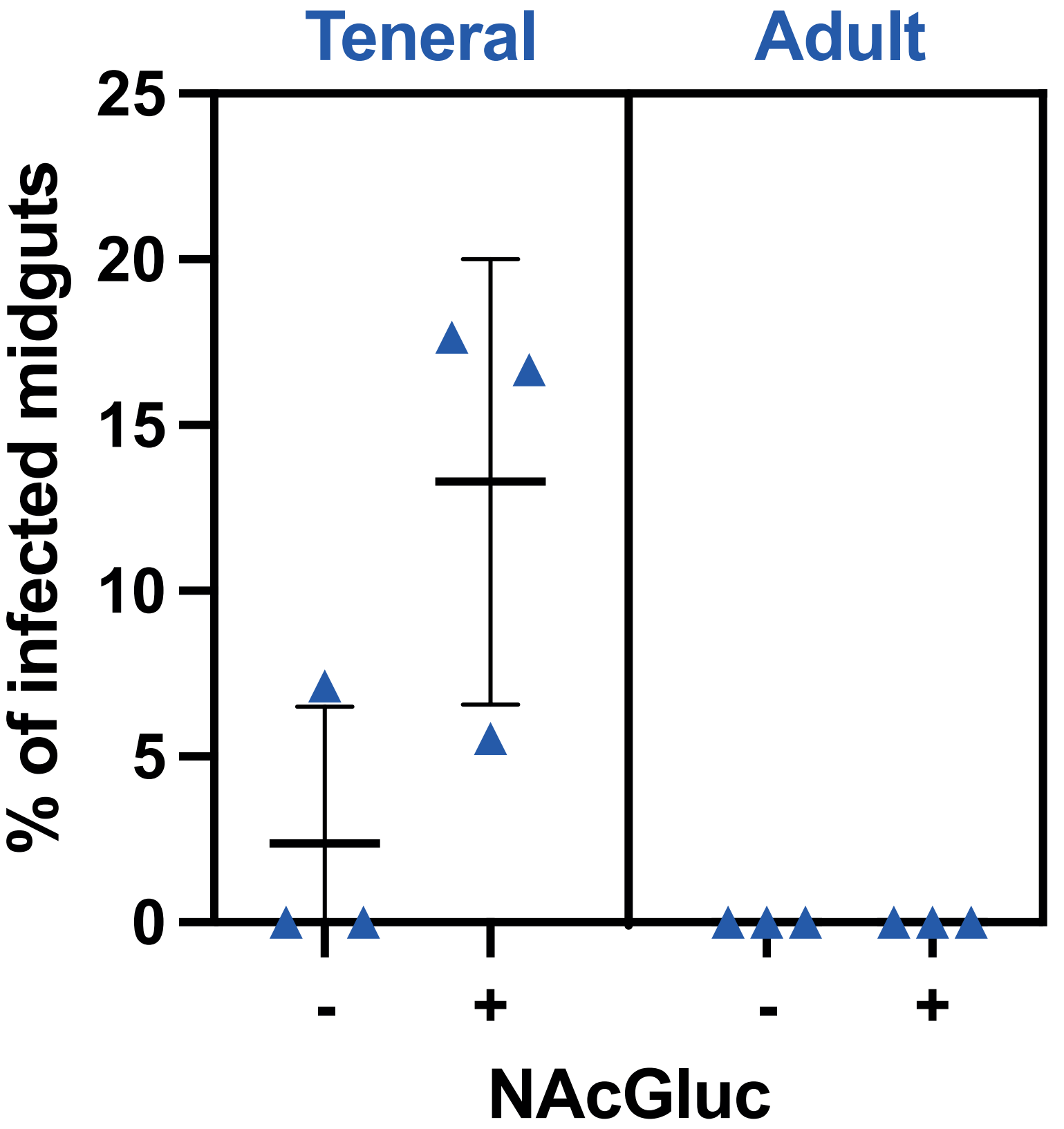
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