1 **The sperm-associated microbiota of crickets and their local variation** 2 **and rapid turnover**

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12 13 **Abstract**

14 While studying aspects of the sperm biology and immunity of two species of crickets, we encountered
15 bacteria that were released from the male sperm container, the spermatophore, alongside sperm. We
16 describe a presumably rich microbe flora in the sperm population ('sperm-associated microbiota').
17 These sperm-associated microbiota differed between the two species of cricket and between different
18 populations and showed functional diversity. Further, sperm-associated microbiota killed sperm,
19 highlighting their potential role in fitness, especially since they are most likely transferred to females
20 during mating.

21 **Introduction**

22 While studying aspects of the sperm biology and immunity of cricket species using the *in vitro*
 23 ejaculation technique (Figure 1A) combined with microbiological standard techniques, we
 24 encountered bacteria that were released from the male sperm container, the spermatophore, alongside
 25 sperm (Figure 1B). After extracting the spermatophore from the male, sterilizing its outside by
 26 drawing it through a flame or by ethanol, we cultivated microbes on agar plates and identified them
 27 (Supplementary Material). Examining these microbes in further detail, we discovered that i) microbes
 28 are regularly present in the spermatophore of crickets. We propose that this warrants the
 29 characterization as sperm-associated microbiota (SAM). ii) The current data suggest rich and diverse
 30 SAM. With six different and independent observation trials involving nine cricket populations (Table
 31 1) and several protocols, we were able to characterize 65 morphotypes based on the external
 32 morphology of microbial colonies. It is important to note here that the same bacterial species may
 33 produce slightly different morphotypes across trials, protocols or other conditions. As expected, we
 34 isolated more unique 16S sequences from these 65 morphotypes, a total of 98 (Table 1). In addition
 35 to taxonomic diversity, the SAM were also functionally diverse. For example, we found differences
 36 in DNase, lactase, lipase and protease activity (K. Reinhardt, unpublished data). iii) Importantly,
 37 using the sperm viability kit, we show that the microbe community isolated from the sperm container
 38 increased sperm mortality in the species or population they were sampled from (Fig 1) although as
 39 yet we are unable to assign the effect to any taxonomic or functional group.

40 iv) The SAM of crickets vary between populations, both in the field and the laboratory. One species,
 41 *Gryllus campestris*, displayed differences between two field sites near Tübingen (Germany) (ca. 4.5
 42 km apart) and between two field sites near Dresden (Germany) (ca. 30 km apart). *Gryllus*
 43 *bimaculatus*, when obtained from different populations of commercial laboratory animal suppliers,
 44 also showed differences in their SAM (Table 1/ Fig. 1). We then revealed that at least some bacteria
 45 in the SAM are of environmental origin: Males brought into the laboratory and kept there for repeated
 46 sampling, showed a tendency in both abundance and species composition to converge towards highly

47 similar SAM, and this notion was true for the two cricket species we investigated (Fig 1). Our attempts
 48 to clarify the infection route were not successful so far.

49 v) Within populations, males varied in the incidence, abundance and composition of the SAM (Table
 50 1/ Fig 1). For example, in 56% of males (35 out of 63) we did not succeed in extracting cultivable
 51 bacteria. However, currently it is not clear to what extent this variation in incidence is related to host
 52 genetic variation or to environmental variation (such as the laboratory (see iii) above, Fig. 1D, E).

53 We have not been able to systematically compare SAM in the spermatophore with that found in the
 54 sperm storage organs of females. However, our observations support the view that the existence of
 55 SAM may be the rule rather than the exception (Altmäe et al. 2019; Rowe et al. 2020). The high
 56 incidence of, and sperm mortality (Fig. 1E) caused by, sperm microbes that we discovered in our
 57 series of natural history observations suggest that the SAM is likely a significant selection factor that
 58 make the broad ecological significance put forward by these reviews plausible. Here, we briefly
 59 discuss how our observations may provide additional interpretations of biological phenomena in four
 60 areas of ecology and evolution - sexual selection, local adaptation, reproductive isolation, and
 61 ecological immunology.

62 Ecological immunology has shown that systemic immune responses or infection affect reproduction
 63 negatively (Table 2; Schwenke et al. 2016)) and, *vice versa*, that mating and reproduction influence
 64 immunity. However, the immunological consequences of mating and reproduction are not consistent
 65 across species, and neither are they across the types of immune responses (Schwenke et al. 2016, Oku
 66 et al. 2019). Moreover, studies frequently mention that effect of possible but unknown sexually
 67 transmitted microbes may contribute to mating-induced variation in immunity (Shoemaker et al.
 68 2006; Schwenke et al. 2016). If SAM are as common in other animals as they are in crickets, they
 69 might be those long-sought after sexually transmitted microbes. Moreover, if SAM occur regularly,
 70 the idea of trade-offs between reproduction and immunity may need additional discussion because
 71 the management of regular sexually transmitted microbes requires a simultaneous investment in
 72 reproduction and immunity (Barribeau and Otti 2020).

Figure 1. The sperm microbiome of crickets. Spermatophores (sperm containers) of male crickets can be used for in-vitro ejaculation on a microscope slide (A), showing a separation between sperm that leaves first, and the seminal fluid. The ejaculate contains microbes which differ between the cricket species and between different populations within species (B, field population 3 and 4). The bacteria are, at least partly of environmental origin: males of two populations, brought to the laboratory and sampled repeatedly, display increasingly similar sperm microbiome (M1-M15 represent different colony morphotypes, the circles and numbers represent the number of individuals in which this morphotypes was found, A1/B1 represents the first spermatophore, A2/B2 the second, and B3 the third spermatophore taken from a male?) in *G. campestris* (C) and *G. bimaculatus* (D). Bacteria, cultivated from the ejaculate of *G. bimaculatus* laboratory populations (lab population 1 and 2), increased sperm mortality in both laboratory populations (n=28-30) in comparison to sperm in a sterile control solution (E) (see supplement for method).

87 **Table 1. List of bacteria detected in the SAM of two cricket species (*Gryllus spec.*). Bacterial**
 88 identity is taken from 16S sequencing of bacteria (see supplement).

cricket species	population	bacteria order	bacteria family	bacteria genus
<i>G. bimaculatus</i>	commercial breeder 2	<i>Lactobacillales</i>	<i>Aerococcaceae</i>	<i>Aerococcus</i>
<i>G. campestris</i>	field population 4	<i>Lactobacillales</i>	<i>Aerococcaceae</i>	<i>Aerococcus</i>
<i>G. bimaculatus</i>	commercial breeder 1	<i>Bacillales</i>	<i>Bacillaceae</i>	NA
<i>G. bimaculatus</i>	commercial breeder 2	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
<i>G. bimaculatus</i>	lab population 1	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
<i>G. campestris</i>	field population 3	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
<i>G. campestris</i>	field population 4	<i>Bacillales</i>	<i>Bacillaceae</i>	<i>Bacillus</i>
<i>G. campestris</i>	field population 4	<i>Hyphomicrobiales</i>	<i>Brucellaceae/Phyllobacteriaceae</i>	NA
<i>G. bimaculatus</i>	lab population 1	<i>Actinomycetales</i>	<i>Dermabacteraceae</i>	<i>Brachybacterium</i>
<i>G. bimaculatus</i>	lab population 1	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Enterobacter</i>
<i>G. bimaculatus</i>	lab population 1	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Enterococcus</i>
<i>G. bimaculatus</i>	lab population 1	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Klebsiella</i>
<i>G. bimaculatus</i>	lab population 2	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Enterococcus</i>
<i>G. bimaculatus</i>	lab population 3	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Enterococcus</i>
<i>G. campestris</i>	field population 3	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Enterococcus</i>
<i>G. campestris</i>	field population 4	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Enterococcus</i>
<i>G. campestris</i>	field population 1	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Enterococcus</i>
<i>G. campestris</i>	field population 1	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Klebsiella</i>
<i>G. campestris</i>	field population 2	<i>Enterobacterales</i>	<i>Enterobacteriaceae</i>	<i>Klebsiella</i>
<i>G. bimaculatus</i>	commercial breeder 1	<i>Pseudomonadales</i>	<i>Moraxellaceae</i>	NA
<i>G. bimaculatus</i>	lab population 3	<i>Pseudomonadales</i>	<i>Moraxellaceae</i>	<i>Acinetobacter</i>
<i>G. campestris</i>	field population 4	<i>Rhodospirillales</i>	<i>Rhodospirillaceae</i>	NA
<i>G. bimaculatus</i>	lab population 1	<i>Bacillales</i>	<i>Staphylococcaceae</i>	<i>Staphylococcus</i>
<i>G. bimaculatus</i>	lab population 1	<i>Bacillales</i>	<i>Staphylococcaceae</i>	<i>Staphylococcus</i>
<i>G. bimaculatus</i>	lab population 2	<i>Bacillales</i>	<i>Staphylococcaceae</i>	NA
<i>G. bimaculatus</i>	lab population 3	<i>Bacillales</i>	<i>Staphylococcaceae</i>	<i>Staphylococcus</i>
<i>G. campestris</i>	field population 3	<i>Lactobacillales</i>	<i>Streptococcaceae</i>	<i>Lactococcus</i>
<i>G. campestris</i>	field population 4	<i>Lactobacillales</i>	<i>Streptococcaceae</i>	<i>Lactococcus</i>
<i>G. campestris</i>	field population 1	<i>Lactobacillales</i>	<i>Streptococcaceae</i>	<i>Lactococcus</i>
<i>G. campestris</i>	field population 2	<i>Lactobacillales</i>	<i>Streptococcaceae</i>	<i>Streptococcaceae</i>
<i>G. bimaculatus</i>	lab population 1	<i>Enterobacterales</i>	<i>Yersiniaceae</i>	<i>Serratia</i>

90 Female investment into immunity likely depends on the composition of the SAM and the probability
 91 of sexual transmission. It is, therefore, possible that SAM assist in explaining differences between
 92 populations and species in the immunological consequences of mating. We believe it is a large,
 93 unexplored area to examine whether female defense against sexually transmitted microbes occurs by
 94 immune upregulation in anticipation to mating (Siva-Jothy et al. 2019) or by constitutive immune
 95 effectors. Future work in ecological immunology may, therefore, benefit from measuring immune
 96 responses just before as well as after mating.

97 The reproductive biology of crickets has long been a model system of behavioral ecology, condition-
 98 dependence and sexual selection research (Zuk and Simmons 1997). Briefly, as soon as the
 99 spermatophore is formed and deposited at a special pouch at the end of the abdomen, males start
 100 acoustic signaling to attract females. For mating, females place themselves over the male and from
 101 underneath the male attaches the spermatophore to the genital opening of the females. A long rod
 102 protruding from the spermatophore (Fig. 1A) is inserted into the female genital opening. Once the
 103 spermatophore is attached, sperm pumps out and travels into the female reproductive tract and sperm
 104 storage organ. While this is happening the male physically separates from the female and stays close
 105 by the female, a behavior known as guarding. Across several species of crickets, mate guarding delays
 106 the time at which the female removes the spermatophore and so increases the number of sperm
 107 migrating into the female. Several sexual-selection related hypotheses have been proposed for both
 108 the spermatophore removal by the female, as well as the guarding duration by the male (Zuk and
 109 Simmons 1997) (Table 2). However, necessarily all of them were tested in the absence of the
 110 knowledge of the existence of SAM reported here. Given that microbes associated with sperm cause
 111 sperm mortality (Fig. 1F), our observation may pave the way to the testing of additional hypotheses,
 112 some of which are proposed in Table 2.

113 We observed for both cricket species that microbes increased sperm mortality, that the SAM have an
 114 environmental component, and that SAM also vary within host species. The three observations
 115 together may allow for the hypothesis that males adapt to the sperm mortality imposed by the local

116 microbes. If so, this would represent a form of local adaptation and represent a significant step
 117 towards any microbially-induced reproductive isolation proposed by Rowe et al. (2020). If such male
 118 adaptation would include the adaptation of ejaculate components (Otti et al. 2013), crickets and their
 119 SAM would represent a promising model of a possible coevolution between seminal fluid
 120 components and locally adapted, sexually transmitted microbes. This idea could have an influence on
 121 the interpretation of paternity outcomes in sperm competition trials involving males from the female's
 122 own or separate population and may also help to explain conspecific sperm precedence in crickets
 123 (Gregory and Howard 1994), and perhaps other species. Because the SAM had an environmental
 124 component, the idea of sperm or ejaculate adaptation to SAM would allow for the prediction that
 125 ecologically more similar habitats harbor more similar sperm microbe communities. Therefore, SAM
 126 might even contribute to ecological speciation (Nosil 2012). Given the microbiota changes in the
 127 laboratory (Fig 1 D, E), our observations are, finally, a plea for field work.
 128

129 **Table 2.** Selection of possible additional or alternative interpretations of cricket reproductive behaviour, immunity and sexual selection in the light of
130 the existence of sperm-associated microbiota.

Observation	Current interpretations	Possible alternative or additional interpretations
Removal of the spermatophore by females is related to male quality (Zuk and Simmons 1997)	Cryptic female choice for male quality based on male or sperm quality	Females prevent the immigration of sperm-associated microbes
Mating changes female immune response (Oku et al. 2019, Schwenke et al. 2016)	(1) Immune suppression protects sperm from the female immune system; trade-off between immunity and reproduction. (2) Immune activation viewed as a reduction of disease transmission risk.	More specifically: Immune activation protects females from infection by sperm-associated microbes.
Mating enhances parasite resistance (Shoemaker et al. 2006)	(1) females receive male compounds that enhance disease resistance (i.e. direct benefits) (2) mating cues females to up-regulate immunity to respond to disease transmission	The female immune system has been upregulated to fight sperm-associated microbes.
Seminal fluid composition is very diverse (Simmons et al. 2013), contains prostaglandin synthetase (Larson et al. 2012)	Increased prostaglandin synthesis in females increases fertilization or egg-laying rates beyond female optimum/ females evolve resistance/ new male substances are selected	Increased prostaglandin synthesis in females increases antibiotic sperm protection from sexually transmitted microbes
Females frequently mate with the same male (Rodríguez-Muñoz et al. 2010)	Females receive fresh sperm (Reinhardt 2007)	Females may reduce the between-male diversity of sexually transmitted microbes
Many females in the wild leave no offspring (Rodríguez-Muñoz et al. 2010)	none	Microbe-induced sperm mortality affects fecundity

References

- Altmäe, S., J. M. Franasiak, and R. Mändar. 2019. The seminal microbiome in health and disease. *Nature Reviews Urology* 16:703–721.
- Barribeau, S. M., and O. Otti. 2020. Sexual Reproduction and Immunity. *eLS*:1–10.
- Gregory, P. G., and D. J. Howard. 1994. A post-insemination barrier to fertilization isolates two closely related ground crickets. *Evolution* 48:705–710.
- Larson, E. L., J. A. Andrés, and R. G. Harrison. 2012. Influence of the Male Ejaculate on Post-Mating Prezygotic Barriers in Field Crickets. *PLoS ONE* 7.
- Nosil, P. 2012. *Ecological speciation*. Oxford University Press, New York.
- Oku, K., T. A. R. Price, and N. Wedell. 2019. Does mating negatively affect female immune defences in insects? *Animal Biology* 69:117–136.
- Otti, O., A. P. McTighe, and K. Reinhardt. 2013. *In vitro* antimicrobial sperm protection by an ejaculate-like substance. *Functional Ecology* 27:219–226.
- Reinhardt, K. 2007. Evolutionary consequences of sperm cell aging. *The Quarterly Review of Biology* 82:375–393.
- Rodríguez-Muñoz, R., A. Bretman, J. Slate, C. A. Walling, and T. Tregenza. 2010. Natural and sexual selection in a wild insect population. *Science* 328:1269–1272.
- Rowe, M., L. Veerus, P. Trosvik, A. Buckling, and T. Pizzari. 2020. The Reproductive Microbiome: An Emerging Driver of Sexual Selection, Sexual Conflict, Mating Systems, and Reproductive Isolation. *Trends in Ecology and Evolution* 35:220–234.
- Schwenke, R. A., B. P. Lazzaro, and M. F. Wolfner. 2016. Reproduction-Immunity Trade-Offs in Insects. *Annual Review of Entomology* 61:239–256.
- Shoemaker, K. L., N. M. Parsons, and S. A. Adamo. 2006. Mating enhances parasite resistance in the cricket *Gryllus texensis*. *Animal Behaviour* 71:371–380.
- Simmons, L. W., Y. F. Tan, and A. H. Millar. 2013. Sperm and seminal fluid proteomes of the field cricket *Teleogryllus oceanicus*: Identification of novel proteins transferred to females at mating.

Insect Molecular Biology 22:115–130.

Siva-Jothy, M. T., W. Zhong, R. Naylor, L. Heaton, W. Hentley, and E. Harney. 2019. Female bed bugs (*Cimex lectularius* L) anticipate the immunological consequences of traumatic insemination via feeding cues. Proceedings of the National Academy of Sciences of the United States of America 116:14682–14687.

Zuk, M., and L. W. Simmons. 1997. Reproductive strategies of the crickets (Orthoptera: Gryllidae). The evolution of mating systems in insects and arachnids 89:109.

Supplementary material

Isolation of microbes

The spermatophores were removed from the male genital opening using forceps. They were sterilised on the outside surface with ethanol or by flame treatment, placed in sterile medium using sterile forceps and crushed (Supplementary Table 1). After incubation, the medium was spread on sterile agar plates. Growing colonies were described morphologically and single bacterial strains were isolated and collected for 16S Sanger sequencing. DNA was isolated from these strains according to the respective protocol of the kit (see Supplementary Table 1), PCR were conducted to amplify variable regions of the 16 S DNA (see text below for details) and the PCR products were sent to a company for sequencing (see Supplementary Table 1). We used BLAST to determine the sequence identity of our bacteria samples in the NCBI database and assigned genus (94.5% sequence identity), family (86.5% sequence identity) and order (82.0% sequence identity) (Yarza et al. 2014). Note that only cultivable and (facultative) aerobic bacteria species can be detected by this method.

Supplementary Table 1 – Details of the different protocols used for the isolation of sperm-associated bacteria.

Study	Sterilisation of outside surface of spermatophore	Growth medium, time, and temperature	Agar, time and temperature	DNA extraction kit	PCR primer	Sequencing
2009 lab population 1	10s in ethanol	Grace's insect medium, 4 h at 30° C	LB agar, 24 h at 30° C	MoBio UltraClean kit	27f (5'-AGAGTTTGATCMTG GCTCAG)	Source Bioscience Geneservice (Nottingham, UK)
2013 lab population 2 & 3; field population 1 & 2	Pulled through flame	Tryptic Soy Broth (TSB), 4 h at room temperature	TSB agar, 24 h at room temperature	Type-it® (QIAGEN)	forward primer 5'-GAAGAGTTTG...GCTCAG reverse primer 5'-ACGACAGCCA...GCA CCT	Macrogen Europe (Amsterdam, The Netherlands)
2019 commercial breeder 1 & 2; field population 3 & 4	Pulled through flame	Brain Heart Glucose Broth, overnight at 29° C	Columbia-Agar with 5% sheep blood, overnight at 37° C	innuPREP DNA Mini Kit (analytikjena)	515f (5'-GTGCCAGCMGCCGCGGTAA) 806r (5'-GGACTACHVHHHTWTCTAAT)	Macrogen Europe (Amsterdam, The Netherlands)

184 **Sperm viability assay**

185 **Bacterial solution.** We made a bacterial solution for two cricket populations from commercial
 186 suppliers. Each bacterial solution contained the SAM isolated from ten spermatophore.
 187 Work was done with sterile instruments in a sterile environment. Males of each population were
 188 randomly chosen. Spermatophores were removed and sterilised on the outside by drawing them
 189 through a flame. To control for contamination from the outside of the spermatophores, they were
 190 each washed in 50 µl brain heart glucose broth (BHG) (Thermo Scientific) on a microscopic slide.
 191 Then, they were placed in a 1.5 ml Eppendorf tube with 500 µl brain heart glucose broth and
 192 crushed. 10 µl of the control were spread on a Columbia agar plate with 5% sheep blood. The
 193 solution with the spermatophores and the plates were incubated at 29°C overnight. Then, the
 194 bacterial solutions produced from ten spermatophores with no bacterial growth on the plates
 195 controlling for contamination were mixed. . The bacterial solutions were stored in the fridge to
 196 inhibit bacterial growth during the experimental period. To achieve comparable conditions for both
 197 populations, the bacterial solutions were adjusted to an optical density of 0.3 A at 600 nm. During
 198 the experimental period, the bacterial density was controlled and adjusted at two-hour intervals.

199 **Sperm viability assay.** We used each 15 males of two laboratory populations of *G. bimaculatus*.
 200 Two spermatophores of each male were sampled and assigned to the control treatment and the
 201 bacterial treatment in randomised order. Spermatophores were removed from males and placed in 7
 202 µl BHG on a microscopic slide. The spermatophore was opened to initiate the release of sperm.
 203 After ten minutes of sperm release, we removed the spermatophore and added 1.5 µl SYBR[®]14
 204 and 2 µl propidium iodide to stain live cells fluorescent green and dead cells fluorescent red. After
 205 two minutes of staining, we added either 10 µl sterile BHG (control) or 10 µl bacterial solution
 206 (bacterial treatment) and measure sperm viability over time as described in Eckel et al, 2017.
 207

208 **Cricket housing**

209 2009: The *G. bimaculatus* population was maintained in transparent plastic boxes at 26°C and a
 210 14:9h light:dark cycle with 30 min dawn/dusk periods. They were fed rat chow (7% cereal (wheat,
 211 maize, barley, wheat- feed) 15% vegetable proteins (soya bean meal), 5% animal protein (fish meal)
 212 and 3% vitamins (major and trace) and amino acids) ad libitum. Water was provided in cotton-
 213 plugged vials.

214 2013: *G. bimaculatus* populations were housed in transparent plastic boxes and *G. campestris* males
 215 were housed in single transparent plastic containers with a normal dark:light cycle corresponding to a
 216 central European summer and at 26°C. They were fed dry cat food (30% protein, 10% fat, 6.5% ashes,
 217 3.5% crude fibre, 1.2% calcium, 1% phosphor and 10 mg/kg copper, 65 mg/kg zinc, 2 mg/kg iodide, 0.2
 218 mg/kg selenium, 1000 mg/kg taurine and 15000 IE/kg vitamin A, 1500 IE/kg vitamin D3 and 150
 219 mg/kg vitamin E) and oat flakes (13.5 g proteins, 58.7 g carbohydrates, thereof 0.7 g sugar, 7.0 g fat,
 220 thereof 1.3 g saturated fatty acids, 10.0 g dietary fibre and <0.01 g natrium) ad libitum. Water was
 221 provided in cotton-plugged vials.

222 2019: *G. bimaculatus* populations were housed in transparent plastic boxes and *G. campestris* males
 223 were housed in single transparent plastic containers at 20°C-25°C and 24 h light. They were fed dry cat
 224 food (32% protein, 15% fat, 6.5% raw ash, 5% crude fibre, 8% moisture content) ad libitum. Water
 225 was provided as aqua pearls with vitamin D (Hobby Terraristik, 1733).

226 **References**

- 227 Eckel, B. A., R. Guo, and K. Reinhardt. 2017. More pitfalls with sperm viability staining and a
228 viability-based stress test to characterize sperm quality. *Front. Ecol. Evol.* 5:165. Frontiers.
- 229 Yarza, P., P. Yilmaz, E. Pruesse, F. O. Glöckner, W. Ludwig, K.-H. Schleifer, W. B. Whitman, J.
230 Euzéby, R. Amann, and R. Rosselló-Móra. 2014. Uniting the classification of cultured and
231 uncultured bacteria and archaea using 16S rRNA gene sequences. *Nat. Rev. Microbiol.* 12:635–
232 645.

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