Immune stimulation alters chemical profiles of adult *Tribolium castaneum*

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1. Abstract

Rapid recognition of disease cues is essential for preventing pathogenic infections and for disease management in group-living animals. Healthy individuals across taxa can detect illness in other conspecifics and adjust their responses to limit further infections of themselves and the group. However, little is known about potential changes in chemical phenotypes due to disease, which may mediate these responses. We here asked whether individual immune experience resulting from wounding or the injection of heat-killed bacteria of *Bacillus thuringiensis* (i.e., immune priming) leads to changes in the chemical profiles of adult red flour beetles (*Tribolium castaneum*). This group-living insect species is a well-studied example for both immune priming as a form of innate immune memory and niche construction via 'external immunity', i.e., the secretion of quinone-containing antimicrobials into the flour. Upon interaction with wounded conspecifics, naive beetles were previously found to not only up-regulate immunity, but moreover reduce gene expression of the evolutionary capacitor HSP90, an effect that has the potential to enhance adaptability. We here used gas chromatography-flame-ionisation detection (GC-FID) to study the composition of stink gland secretions and cuticular hydrocarbons (CHCs) of immune-primed and wounded beetles compared to controls. The overall profiles as well as target compounds of the stink gland secretions showed transient, slight changes after these treatments, particularly in wounded females. Priming and wounding led to pronounced changes in CHC profiles with increases in the proportion of methyl-branched alkanes. Furthermore, we found sex-specific differences, that were particularly pronounced in the CHCs, although the changes due to immune stimulation were overall similar in both sexes. We suggest that CHCs are potential candidates for the transfer of information related to individual immunological experience into the group.

Keywords

immune priming, innate immunity, disease cues, cuticular hydrocarbons, GC-FID, niche construction, stink glands, *Tribolium castaneum, Bacillus thuringiensis*

2. Introduction

The ability to recognise the health-status of others is vital for decision making and the success of individuals. This is especially true in animal groups where increased contacts and host density facilitate the spread of diseases (Kappeler et al., 2015). Healthy individuals in many species were shown to modulate their behaviour and physiology in response to disease (Lopes, 2020), by either avoiding harmful microbes and diseased individuals (Arakawa et al., 2011; Schulenburg & Ewbank, 2007; Stensmyr et al., 2012) or managing active infections once the pathogen had entered the group. The best-studied examples are found in eusocial insects (Cremer et al., 2018) that show remarkable group-level disease defences such as social distancing (Stockmaier et al., 2021), hygiene and sanitary care (Konrad et al., 2012; Rosengaus et al., 1998) or social immunisation (Masri & Cremer, 2014). However, still very little is known about the effects of immune status on chemical phenotypes which may mediate these responses.

Importantly, to enable individuals to respond to pathogenic threats and to adjust their reactions, they need to be able to detect disease cues in others. Chemical cues have been shown to allow healthy animals to identify diseased conspecifics across taxa (Behringer et al., 2020; Csata et al., 2017; Lopes, 2020; Pull et al., 2018). For example, eusocial insects can detect immunological and disease cues of other nestmates and adjust subsequent social responses towards diseased members (Pull et al., 2018; Sun et al., 2017). In honey bees, these responses lead to the modulation of social interactions among workers (Baracchi et al., 2012; Mondet et al., 2021; Richard et al., 2008) as well as immune activation in queens (Hernández López et al., 2017). Many of these reactions were shown to be mediated, among other compounds, by changes in cuticular hydrocarbon profiles (CHCs). CHCs are present on the surface of insects and consist of complex mixtures of straight-chain, methyl-branched, and unsaturated hydrocarbons (Blomquist & Ginzel, 2021). Their content and composition can vary with age, diet, development stage, sex, temperature, and geographic origin of species or

68 populations (Blomquist & Bagnères, 2010). They are involved in a wide range of functions
69 from prevention of desiccation to serving as pheromones or allelochemicals for recognition of
70 species, sex, mutualistic partners, or hosts (Blomquist & Bagnères, 2010; Howard &
71 Blomquist, 2005). CHCs are particularly important in social insects, where they serve for
72 nestmate recognition and many aspects of regulation of the social interactions inside the nests
73 (Leonhardt et al., 2016). Since CHCs are hardly volatile, direct contact is required for
74 recognition of these compounds. In honey bees, immune challenge and wounding lead to
75 changes in CHC profiles in workers, which are then likely recognised by queens upon contact
76 and may result in the immune activation described above (Hernández López et al., 2017).
77 Currently, similar observations are rare in non-social organisms (Gallagher et al., 2018),
78 making it difficult to compare chemical disease communication across animal groups.
79 We have previously shown in the red flour beetle, *Tribolium castaneum* (Coleoptera:
80 Tenebrionidae), which is a non-social, but group-living (gregarious) insect species that lives
81 in and consumes flour, that the interaction of naïve focal beetles with wounded conspecifics
82 induced increased activity of the important immune effector phenoloxidase (PO) (Peuß et al.,
83 2015). In addition to this social transfer of immunity, we also found a consistent reduction in
84 expression of two genes coding for the heat shock protein HSP90, namely *Hsp83* and *Hsp90*,
85 in focal beetles. As the molecular chaperone HSP90 serves as an evolutionary capacitor
86 (Rutherford & Lindquist, 1998), such down-regulation has the potential to lead to increased
87 adaptability via the release of cryptic genetic variation. However, it is up to now unknown,
88 which cues emitted by wounded beetles could be recognised by the naïve focal beetles.
89 Immune priming is a form of innate immune memory in invertebrates (Little &
90 Kraaijeveld, 2004; Milutinović & Kurtz, 2016; Netea et al., 2016; Pham et al., 2007).
91 *Tribolium castaneum* is a prime example for immune priming, which can be specific for the
92 type of pathogen (Roth et al., 2009) and can be transferred from parents to offspring (Roth et
93 al., 2010). In addition to protection achieved by their internal immune system, *T. castaneum*

engages in external immune defences (Joop et al., 2014; Otti et al., 2014), i.e., a process of niche construction (Odling-Smee et al., 2013) mediated by the beetles' stink gland secretions (Sokoloff, 1974). These secretions serve a hygienic function, as they contain the benzoquinones ethyl- and methyl-1,4-benzoquinone (EBQ and MBQ) along with further substances with inhibitory effects on microbes (Li et al., 2013; Yezerski et al., 2007). However, at high concentrations these compounds become harmful to the beetles themselves (Sokoloff, 1974), and might also be involved in female density-dependent competition (Khan et al., 2018). Moreover, biosynthesis of the quinones depends on the availability of tyrosine, a semi-essential amino acid, which is also a substrate for the PO enzyme that is central to internal immune defences of crustaceans and insects (Cerenius et al., 2008). The amount of secretion might therefore be optimised by evolution (Joop et al., 2014) and adjusted to external conditions (Pedrini et al., 2010). In theory, the level or composition of the beetles' secretions inside the flour could be recognised by conspecifics and used for a potential adjustment of production and release of own secretions (Gokhale et al., 2017).

We here examined the influence of individual immune experiences, i.e., wounding and immune priming, compared to controls on the chemical profiles of red flour beetles, particularly with respect to stink gland secretions and CHCs. As a response to wounding and/or priming being a cue for a potential pathogenic threat, we expected to see (1) changes in the profile of the stink gland secretions and in the main compounds, EBQ, MBQ and their carrier 1-pentadecene (Loconti & Roth, 1953; Villaverde et al., 2007); (2) changes in the CHC profiles, which may act as a cue for conspecifics, leading to the previously described changes in immunity and *Hsp*83/*Hsp90* expression . For the former, either higher or lower concentrations could be expected in response to wounding or priming, as an increased contribution to external immunity, or as a result of the trade-off with internal immunity responding to the wounding or priming, respectively. We analysed the chemical profiles for

119 males and females separately, thereby taking into account potential sex differences in CHC

120 profiles and sex-specific functions of the secretions (Khan et al., 2018).

121 **3. Methods**

122 3.1 Model organisms

123 Our study was done on the outbred strain Croatia 1 (CRO1) of *T. castaneum,* which
124 was derived from a wild population originally collected in Croatia, May 2010 (Milutinović et
125 al., 2013). This outbred population was then kept at 30 ºC, 70% humidity and a 12 h:12 h
126 light:dark cycle, in plastic boxes containing heat-sterilised (75 ºC for at least 24 hours)
127 organic wheat flour (Bio Weizenmehl Type 550, dm-drogerie markt) with 5% brewer's yeast
128 powder (hereafter the flour-yeast mixture is referred to as "flour"). For this experiment,
129 around 2,000 one month old adult beetles were allowed to mate and oviposit for 24 h in
130 approximately 350 g flour. Following that, adult beetles were separated from the flour
131 containing eggs using a 710 µm mesh size sieve (Retsch). All beetles were kept individually
132 to minimise any injuries due to conspecific cannibalism or mating (Alabi et al., 2008; Park et
133 al., 1965; Via, 1999).

134 *Bacillus thuringiensis morrisoni* bv. *tenebrionis* (*Btt*) was obtained from Bacillus
135 Genetic Stock Center (Ohio State University, USA) and heat-killed vegetative cells used for
136 the priming treatment were prepared as previously described (Ferro et al., 2017; Roth et al.,
137 2009). In short, 100 µL of a *Btt* freezer stock (stored at -80 ºC) was inoculated into a 500 mL
138 baffled flask containing 50 mL Luria Broth (Carl Roth GmbH + Co. KG) and the culture was
139 incubated at 30 ºC, 200 rpm for 15 h in dark. Subsequently, the culture was transferred into a
140 50 mL Falcon conical tube and centrifuged at 3,645 × g at 4 ºC for 10 min. The bacterial
141 pellet was washed once with phosphate buffered saline (PBS, Calbiochem®) and the
142 concentration adjusted to 1×10^9 cells mL^{-1}. PBS (as injection/wounding control) and *Btt* at 1
143 $\times 10^9$ cells mL^{-1} solution (priming solution) were heated at 95 ºC for 30 min to heat-kill the
144 bacteria. All procedures were carried out under a horizontal laminar air-flow hood to keep the
145 conditions sterile.

146

147 3.2 Immune treatment

148 Ten days after oviposition, all experimental beetles were individually placed into
149 sterile 96-well plates (Type F, SARSTEDT) containing 0.08 g flour per well. One week post
150 eclosion, virgin beetles of both sexes were divided into three treatment groups, i.e. naïve
151 (handling control), wounded (injected with sterile PBS) and primed (injected with heat-killed
152 *Btt*). 18.4 nL (approximately 20,000 cells per injection in the priming treatment) of solution
153 was injected with a nanoinjector (Drummond Nanoject II) into the dorsal vessel of adult
154 beetles by puncturing the epidermis between the head and pronotum laterally towards the
155 abdomen.

156 For preparation of stink gland secretion collection, 48 beetles of each sex underwent
157 either of the three treatments (naïve, wounded and primed) and were sampled twice, after 24 h
158 and 72 h, resulting in n = 8 samples of individual beetles per sex, treatment and time point.
159 Handling stress during the injection process can trigger the release of stink gland contents
160 (Lai Ka Lo's personal observation). Therefore, sampling time points of 24 h and 72 h post
161 treatment were chosen to allow the stink gland contents to replenish. After the treatment,
162 beetles for sampling after 24 h were transferred into clean 96-well plates for starvation to
163 reduce the contamination of samples by faecal material and flour. Beetles to be sampled after
164 72 h received flour in well-plates until 24 h before extraction.

165 For preparation of profiling of CHCs, 72 beetles of each sex underwent either of the
166 three treatments, resulting in n = 6 samples per sex and treatment with four beetles pooled
167 into each sample. The time point of the sampling was chosen to be 18 h based on a previous
168 study on *T. castaneum* (Peuß et al., 2015) that reported transfer of immunity, as well as a
169 downregulation of the evolutionary capacitor Hsp90, both of which were mediated by social
170 cues from conspecifics. This downregulation was strongest at the 18 h time point. After
171 treatment, beetles were transferred into clean 96-well plates for starvation for 18 h until
172 sampling.

173

174 3.3 Extraction of beetle chemicals

175 For extraction of glandular secretions, beetles were transferred to individual 1.5-mL
176 safe-lock tubes (Eppendorf). These were immersed in ice water at 3 °C for 3 min to stimulate
177 the release of stink gland secretions (Joop et al., 2014) and then transferred to a -20 ºC freezer
178 to kill the beetles. 65 µL ice-cold acetone (LC-MS grade, Sigma-Aldrich) containing 0.05 mg
179 mL^{-1} octadecane (98.5%, Sigma-Aldrich) as an internal standard was added to each frozen
180 sample Subsequently, the tubes were shaken at 4 °C for 5 min at 1,000 rpm (Thermomixer
181 comfort, Eppendorf) to extract the quinone-rich stink gland secretions. The extracts were
182 centrifuged for 5 min at 16,100 × g at 5 ºC. Subsequently, 50 µL of each extract was
183 transferred into brown vials (FisherBrand, Fisher Scientific) and kept at -20 ºC until analysis.
184 Together with the beetle samples, four blank samples per time point were prepared that
185 underwent the whole extraction process but did not contain beetles.

186 For extraction of the CHCs, four beetles were pooled in 2-mL safe-lock tubes and
187 killed at -20 ºC. The dead beetles were transferred to new 1.5-mL tubes to exclude eventually
188 released stink gland secretions stuck to the tube surface. To each sample, 120 µL of *n*-hexane
189 (GC-MS grade, Merck) and subsequently 5 µL of eicosane (99.5%, Sigma-Aldrich) solution
190 (0.2 mg mL^{-1} in *n*-hexane) as internal standard were added. After shaking at 4 ºC for 10 min,
191 the samples were centrifuged for 5 min at 16,100 × g and 4 ºC. 100 µL of the supernatant
192 containing the surface extracts were transferred to fresh 1.5 mL tubes without touching the
193 beetles at the bottom. The extract was dried under reduced pressure for 5 min at room
194 temperature to somewhat reduce the amount of dominant stink gland secretion compounds
195 that are not stable to drying. Finally, 80 µL of *n*-hexane was added to resolve the dried extract
196 by shaking at 1,000 rpm for 10 min, which was then transferred to transparent vials
197 (FisherBrand) for the chemical analysis. The volume of n-hexane was scaled down
198 accordingly in three samples where less than four beetles were available. Together with the

199 beetle samples, four blank samples were prepared that underwent the whole extraction process
200 but did not contain beetles.
201
202 3.4 Chemical analysis, data pre-processing and feature identification
203 All samples were analysed on a gas-chromatograph coupled with a flame ionisation
204 detector (GC-2010 Plus, Shimadzu) and equipped with a VF-5ms column (30 m × 0.25 mm ×
205 0.25 µm, with 10 m EZ-guard column, J&W Agilent Technologies) and an autosampler
206 (AOC-20i, Shimadzu). For the analysis of stink gland secretions, a sample volume of 1 µL
207 was injected with a split ratio of 3 at a constant stream of hydrogen flow of 1.13 mL min^{-1}.
208 The column oven temperature was set at 50 ºC, held for 2 minutes and increased to 300 ºC
209 with a rate of 10 °C min^{-1}, followed by a holding time of 10 min. For the analysis of CHCs, 1
210 µL from each sample was injected with a split ratio of 2 at a constant nitrogen flow of 1.02
211 mL min^{-1}. The column oven temperature started at 100 °C, first increased to 230 °C with a
212 rate of 20 °C min^{-1}, then increased to 260 °C with a rate of 5 °C min^{-1}, further increased to
213 277 °C with a rate of 1 °C min^{-1}, and finally increased to 320 °C with a rate of 5 °C min^{-1},
214 which was held for 9.9 min. Additionally to the samples and blanks, two alkane mix
215 standards, C8-20 and C8-C40 (both Sigma-Aldrich), were analysed using both
216 chromatographic methods. Furthermore, commercially available standards of MBQ (98%
217 purity), methyl-1,4-hydroquinone (99%, both Sigma-Aldrich) and ethyl-1,4-hydroquinone
218 (97%, Abcr) were analysed using the method for stink gland secretions.
219 Data pre-processing was done using the GCsolution Postrun software (Version
220 2.30.00, Shimadzu), exporting peak areas with at least 5 (stink gland secretion) and 100
221 counts (CHCs), respectively. Retention time alignment of peaks was done in R (version 4.0.3,
222 R Core Team, 2020) using the package *GCalignR* (Ottensmann et al., 2018), allowing a
223 maximum shift in retention time by 0.05 min. Features that could not be distinguished from
224 those occurring also in blank samples were excluded. The resulting datasets were both

225 normalised to the internal standard and only features detected in at least half of the replicates
226 per treatment and sex (and time point in case of stink gland secretions) were considered for
227 further analysis. In both datasets, relative values were calculated by expressing the amount of
228 a certain feature as the proportion of the total amount of features considered for a sample.
229 For feature identification, the retention times of peaks from the alkane mix standards
230 were used to calculate the retention index (RI) of detected features according to Kováts
231 (1958). From the stink gland analysis, benzoquinones (BQs) as well as other major
232 compounds previously described in *T. castaneum* glandular secretions were identified by
233 comparing their RIs to previous studies (Lehmann, 2015; Li et al., 2013), and by comparisons
234 to the commercial standards analysed. Putative identification of CHCs was done by
235 comparing the RIs with those published in studies by Alnajim et al. (2019), Awater-Salendo
236 et al. (2020) and Lockey (1978).
237
238 3.5 Statistical analyses
239 All statistical analyses were done using R (version 4.0.3, R Core Team, 2020). To
240 visualise dissimilarities between samples of naïve, wounded and primed beetles, non-metric
241 multidimensional scaling (NMDS) was applied to all datasets using the *metaMDS* function in
242 the package *vegan* (version 2.5-7, Oksanen et al., 2020). For this, datasets were transformed
243 using the Wisconsin double standardisation (package *vegan*) of the square root values and
244 pairwise dissimilarities were calculated based on the Kulczynski index. NMDS analysis was
245 done on relative feature abundances, separately for the two time points for the analysis of
246 stink gland secretions, and in all cases performed with both sexes included and also for both
247 sexes separately. For the CHC profiles, naïve males and females were also compared in a
248 separate analysis to visualise general CHC profile differences attributed to sex. Additionally
249 to NMDS, permutational multivariate analysis of variance (PERMANOVA) was performed
250 using the *adonis* function (package *vegan*) to test for the influence of treatment, sex and the

interaction of these factors (only in cases were both sexes were included) on the dissimilarities displayed by the datasets. The same transformations and dissimilarity indices as for NMDS were employed and 10,000 permutations were used. Prior to the PERMANOVA, the *betadisper* and *permutest* (10,000 permutations) functions (package *vegan*) were used to confirm the homogeneity of the multivariate variance spread among the treatment groups. If significant treatment effects were found in the PERMANOVA analyses of CHCs of females and males separately, the *pairwiseAdonis* function (Martinez Arbizu, 2020) was used for post hoc tests with correction of p-values due to multiple comparisons between treatment groups using the Benjamini-Hochberg (Benjamini & Hochberg, 1995) procedure (a = 0.05).

For the secretions, we first identified features that differed between treatments using a conditional random forest classification (seed 245, n trees = 500, n variables per split = 5) (De Moraes et al., 2014; Strobl, Hothorn, et al., 2009; Strobl, Malley, et al., 2009). The analysis was done on relative feature amounts, since it is difficult to control random gland emptying before and during experimental procedures and hence total amount of secretions may differ between animals for reasons other than experimental treatments. Random forest identified one peak as important for each sex, whose relative concentrations were further analysed in post hoc testing. For this, we also included target compounds with known biological activity - EBQ, MBQ and 1-pentadecene (1-C15-ene) - and tested for the significance of our two main effects, 'treatment' (naive, wounded, primed) and 'sex' (male, female), as well as their interaction by fitting linear models (LM) using the *lm* function from the *base* package in R (R Core Team, 2018). Additionally, for the target features we analysed the absolute values as well, since those features were most abundant. We tested for data normality, heteroscedasticity, presence of multicollinearity and influential cases and obtained diagnostics plots (Q-Q plots and histograms of model residuals distribution and a plot to estimate non-linear patterns of residuals). For all models, we first tested the significance of the overall

model including the main effects and their interaction compared to a null model (intercept only) using *anova* function in R. If the model assumptions were not met, we first tried transforming the data using the package *bestNormalize* (Peterson, 2017), after which a non-parametric Kruskal-Wallis (KW) and post hoc comparisons were performed. Multiple comparison corrections were performed for the overall models (or KW tests) separately within absolute and relative datasets and for post hoc tests for each feature, using the Benjamini-Hochberg procedure (a = 0.05).

To visualise changes of the CHC profiles of males and females due to treatments, log2 fold change values were calculated for all CHCs and replicates in comparison to the median value of the naïve male and female beetles and plotted as heatmaps using the package *pheatmap* (Kolde, 2019). Also, heatmaps comparing CHCs of naïve females and naïve males were generated based on z-scores for each CHC. All heatmap plots included Euclidean cluster analysis of CHCs.

290 **4. Results**

291 4.1 Stink gland secretions

292 In the dataset acquired 24 h after treatment, 26 features were included, of which 24
293 were also considered in the dataset acquired 72 h after treatment (Table S1). The seven most
294 dominant features could be identified by comparison of the RI to the literature and to
295 commercial standards. The two benzoquinones, MBQ and EBQ, could clearly be identified,
296 but the corresponding hydroquinones were not present in the dataset. The other five identified
297 features were alkenes with one or two double bonds, namely 1,6-C15-diene, 1-C15-ene, 1-
298 C16-ene, 1,8-C17-diene and 1-C17-ene (Table S1).

299 The NMDS plots on relative features of this dataset showed only a slight separation of
300 secretion composition between sex and treatment groups (Fig. 1a), which nevertheless
301 revealed statistically significant effects (PERMANOVA; sex × treatment: $F = 0.9$, $R^2 = 0.04$,
302 $p = 0.564$; sex: $F = 2.3$, $R^2 = 0.05$, $p = 0.042$; treatment: $F = 2.4$, $R^2 = 0.10$, $p = 0.015$). For
303 secretions of female beetles alone there was a marginally significant treatment effect
304 (PERMANOVA; $F = 2.0$, $R^2 = 0.17$, $p = 0.057$). In post hoc tests, and also visible in the
305 NMDS plot (Fig. 1b), this effect could be attributed to differences in secretion composition
306 between naïve and wounded females ($F = 4.4$, $R^2 = 0.24$, $p = 0.006$), whereas there were no
307 significant differences between naïve and primed ($F = 0.9$, $R^2 = 0.06$, $p = 0.472$), and between
308 wounded and primed females ($F = 1.4$, $R^2 = 0.09$, $p = 0.365$). Analysing male beetles
309 separately, the relative composition of the stink gland secretion seemed visually quite similar
310 in all three treatment groups (Fig. 1c), and likewise there was no significant treatment effect
311 (PERMANOVA; $F = 1.4$, $R^2 = 0.12$, $p = 0.180$). See Figs S1 and S2 for visualisation of the
312 contribution of individual secretion features to the separation shown in NMDS plots.

313 In the dataset acquired 72 h after treatment, there was only a slight separation of
314 relative secretion composition between sexes but not with regard to treatment groups (Fig.
315 S3a), and accordingly, only a marginally significant sex effect was revealed (PERMANOVA;

316 sex × treatment: $F = 0.2$, $R^2 = 0.01$, $p = 0.989$; sex: $F = 1.9$, $R^2 = 0.04$, $p < 0.092$; treatment: F
317 $= 0.9$, $R^2 = 0.04$, $p = 0.485$). Female and male beetles were nevertheless separately tested for
318 treatment effects but none of those were significant (PERMANOVA; $F = 0.6$, $R^2 = 0.05$, $p =$
319 0.802; males: $F = 0.6$, $R^2 = 0.06$, $p = 0.854$), or visible in NMDS plots (Figs S3b, c). No
320 changes in relative composition of gland secretion features due to treatments were suggested
321 by NMDS and PERMANOVA results; thus, analysis of target features was in the following
322 only done for the dataset acquired 24 h after the treatment.

323 Random forest revealed one unidentified feature as important for each sex (Fig. 2a, b);
324 the relative amount of the feature with RI 1015 was reduced in both primed and wounded
325 females (Kruskal-Wallis test, females: $\chi^2 = 9.84$, df = 2, $p = 0.034$, males: $\chi^2 = 1.72$, df = 2, p
326 $= 0.49$), whereas the feature with RI 1473 was specifically increased only in wounded males
327 (Kruskal-Wallis test, females: $\chi^2 = 3.43$, df = 2, $p = 0.37$, males: $\chi^2 = 9.27$, df = 2, $p = 0.034$).
328 We further examined MBQ, EBQ and 1-C15-ene in more detail because of their known
329 biological role in beetle communication (Verheggen et al., 2007) and as antimicrobial agents
330 (Pedrini et al., 2010; Prendeville & Stevens, 2002; Yezerski et al., 2007). Whereas the relative
331 composition of the three compounds MBQ, EBQ and their carrier 1-C15-ene did not change
332 in relation to treatment and sex (Fig. 3a, c, e; LM; EBQ: $F = 1.071$, df = 5, $p = 0.49$, MBQ: F
333 $= 1.511$, df = 5, $p = 0.37$, 1-C15-ene: $F = 0.69$, df = 5, $p = 0.63$), we found strong treatment
334 and sex differences in their absolute amounts (Fig. 3b, d, f; LM; EBQ: sex × treatment: F =
335 1.26, df = 2, $p = 0.3$, treatment: $F = 4.65$, df = 2, $p = 0.015$, sex: $F = 4.7$, df = 2, $p = 0.036$,
336 MBQ: sex × treatment: $F = 1.69$, df = 2, $p = 0.1975$, treatment: $F = 4.3$, df = 2, $p = 0.02$, sex:
337 $F = 4.58$, df = 2, $p = 0.038$, 1-C15-ene: Kruskal-Wallis test (females) $\chi^2 = 9.75$, df = 2, p =
338 0.017, Kruskal-Wallis test (males) $\chi^2 = 3.28$, df = 2, $p = 0.19$). Although the absolute amount
339 of compounds cannot be precisely controlled during experimental handling (see Methods), we
340 found a consistent increase in absolute amount of all target compounds in wounded females,
341 but this was not statistically different from primed females for EBQ and MBQ (Fig 3b, d).

342 Therefore, our results indicate that relative and absolute composition of glandular secretions
343 in *T. castaneum* change in respect to treatment and sex and that wounding induces different
344 physiological signatures from priming.
345
346 4.2 Cuticular hydrocarbons
347 In total, 20 CHCs were putatively identified in the dataset. These consisted of seven *n*-
348 alkanes (C25-C31), six 3- and 4-methyl-branched alkanes and seven internally methyl-
349 branched alkanes of which one was a dimethyl alkane (Table S2). The RIs of all identified
350 CHCs did not differ by more than ± 4 from the RI values proposed by the literature (Lockey
351 1978; Alnajim et al. 2019; Awater-Salendo et al. 2020). The dominant CHCs in both naïve
352 females and males were *n*-C29, 3-MeC27, 13-MeC27 and *n*-C27, all with a higher proportion
353 than 10% (Table S2).
354 NMDS plots resulted in a clear separation of CHC profiles based on relative
355 composition of males and females and also in a separation of CHC profiles of beetles from
356 different treatments within sexes (Fig. 4a). Accordingly, the influence of these factors, but not
357 of their interaction, on the variation in the dataset was significant (PERMANOVA; sex ×
358 treatment: $F = 0.4$, $R^2 = 0.01$, $p = 0.827$; sex: $F = 60.8$, $R^2 = 0.60$, $p < 0.001$; treatment: $F =$
359 4.5, $R^2 = 0.09$, $p = 0.004$). Scores of individual CHCs on the NMDS plot (Fig. 4b) and
360 heatmaps comparing relative values of CHCs to naïve males and females (Fig. S4) visualised
361 groups of CHCs differentiating beetles by sex. Most obviously, male CHCs harboured higher
362 proportions of rather long-chain *n*- and also methyl alkanes, especially of the cluster
363 comprising 3-MeC29, *n*-C30 and *n*-C31 (Fig. 4b, Fig. S4). In contrast, the CHC profile of
364 female beetles was characterised by higher proportions of rather short-chain methyl alkanes,
365 especially of the cluster comprising 10-/11-/12-MeC26, 3-MeC27 and 13-MeC27 (Fig. 4b,
366 Fig. S4).

367 Regarding females alone, relative CHC profiles of naïve beetles visually differed from
368 those of beetles of both treatments, but separated stronger from CHC profiles of wounded
369 than those of primed beetles (Fig. 4c), which fits the statistically significant treatment effect
370 (PERMANOVA; $F = 2.5$, $R^2 = 0.25$, $p = 0.023$). In post hoc tests, CHC profiles of naïve
371 beetles differed significantly from those of wounded ($F = 4.8$, $R^2 = 0.33$, $p = 0.006$), but not
372 from those of primed beetles ($F = 2.0$, $R^2 = 0.17$, $p = 0.153$), while CHC profiles of wounded
373 and primed beetles were quite similar ($F = 0.6$, $R^2 = 0.06$, $p = 0.630$). As inferred from the
374 CHC scores in the NMDS plot and heatmaps showing treatment-related differences relative to
375 naïve beetles, higher proportions of shorter methyl alkanes (C26-C29) characterised CHC
376 profiles of both wounded and primed females in comparison to those of naïve females (Fig.
377 4c, d). The CHC profiles of naïve females had higher proportions of comparatively longer *n*-
378 alkanes (C29-C31) and also of 3-MeC31 (Fig. 4c, d).
379 CHC profiles of male beetles revealed a comparable picture as those of females in
380 NMDS plots, where profiles of naïve beetles separated from those of wounded and primed
381 ones (Fig. 4e), which was mirrored in a significant treatment effect (PERMANOVA; $F = 2.4$,
382 $R^2 = 0.24$, $p = 0.009$). Post hoc tests revealed similar patterns as in females, with CHC
383 profiles of naïve males differing significantly from those of wounded ($F = 3.8$, $R^2 = 0.28$, $p =$
384 0.009), but not from CHC profiles of primed males ($F = 2.1$, $R^2 = 0.18$, $p = 0.110$), whereas
385 CHC profiles of wounded and primed males did not differ at all ($F = 1.2$, $R^2 = 0.10$, $p =$
386 0.317). The same CHCs as in females were overall involved in separation of naïve from
387 wounded and primed beetles, although the contribution of individual CHCs to that separation
388 slightly differed (Fig. 4e, f).

5. Discussion

As group-living animals that share the same food resources and living space at high population densities, flour beetles are prone to increased transmission of parasites or pathogens via shared food and frequent direct contacts. However, flour beetles alter their environment using stink gland secretions that have a major influence on the microbial load of flour (Sokoloff, 1974; Van Wyk et al., 1959), thereby constructing the niche in which adults and their offspring live in. As some of the secreted substances have antimicrobial activity, the secretions are also considered a form of 'external immunity' (Joop et al., 2014; Otti et al., 2014), which, like an internal immune system, needs to act prophylactically and be adjusted to the anticipated demands. This is especially important from the aspect of parental care (Clutton-Brock, 1991; Trivers, 1972), as adults may modulate their stink gland secretion composition upon detected pathogen risks to protect their offspring, which do not possess these glands. Indeed, in our experiment we found a female-specific increase in the absolute amount of target compounds from stink gland secretions (Fig. 3a, c, e) that was significant only for the wounded, and less so primed females. These results could reflect different reproductive and brood-care investments between females and males, as females invest more resources into immunity and longevity (Bateman, 1948; Rolff, 2002) or into brood protection from parasites (Trivers, 1972). In contrast to the observed change in the absolute amounts of MBQ, EBQ and 1-C15-ene, we found no change in the relative composition of these or other features in our dataset, except in the two yet unidentified sex-specific features (RI 1015 females, RI 1473 males) that were overall represented in lower relative abundance (Fig 1. a, b). Whereas the relative intensity of RI 1015 was lower in both wounded and primed females, feature RI 1473 was relatively higher in wounded males. The two unidentified features of sex-specific relative abundance responding to immune treatments may serve various sex-differential functions as seen in *Tenebrio molitor* such as investment in offspring (Nielsen &

414 Holman, 2012) or attractiveness for mates (Rantala et al., 2002; Sadd et al., 2006; Worden et
415 al., 2000).

416 In our study, wounding revealed to have a more pronounced effect on the chemical
417 composition of stink gland secretions than priming, compared to secretions from naive control
418 beetles (Fig. 3b, d, f). This may result from the specificity of the immune response against the
419 bacterium (here *Btt*) used for priming (Ferro et al., 2019; Roth et al., 2009), whereas
420 compounds from stink gland secretions, being broad in their antimicrobial activity, may
421 provide protection against yet unknown pathogens invading the body cavity, and could thus
422 be a more appropriate response after wounding. For example, BQs are important for
423 protection against various bacteria and fungi (Joop et al., 2014; Pedrini et al., 2015; Sawada et
424 al., 2020), which might gain access to the insects' body cavity after wounding. The observed
425 differences in quinone production may also result from a resource trade-off between internal
426 and external immunity; tyrosine is a limiting resource for both, the PO cascade within the
427 internal immune defence and the production of quinones for secretion (Joop et al., 2014; Otti
428 et al., 2014). The strong and lasting activation of PO shown in *T. castaneum* upon *Bt* infection
429 (Behrens et al., 2014) might compete for tyrosine that is also needed for the production of
430 quinones. The broad and unspecific responses turned on after wounding seem to be costly
431 (Schulz et al., 2019), and some of these costs might be explained by the increased production
432 of antimicrobial secretion compounds.

433 Apart from serving as antimicrobials and thus allelochemicals, BQs and alkenes in
434 stink gland secretions also lead to dose-dependent behavioural responses in *T. castaneum*, i.e.
435 serve as pheromones (Suzuki, 1985; Verheggen et al., 2007). Low amounts of quinones
436 trigger aggregation, whereas high amounts become repellent or even toxic to conspecifics
437 (Chapman, 1926; Roth & Howland, 1941; Sokoloff, 1974). The BQs and alkenes act in
438 concert with an aggregation pheromone, 4,8-dimethyldecanal, and mediate dispersal of
439 conspecifics (Duehl et al., 2011; Verheggen et al., 2007). If wounding, e.g., via intraspecific

440 cannibalism or interspecific competition, stimulates increased secretion of BQs, this can in
441 turn signal overcrowding (Faustini & Burkholder, 1987; Verheggen et al., 2007) and result in
442 further interference competition mediated by BQs (Khan et al., 2018; Sonleitner & Gutherie,
443 1991) or even dispersal (Duehl et al., 2011; Ogden, 1969), thereby influencing group
444 dynamics and population size. Because flour conditioning and chemical cues affect the
445 development, behaviour and survival of beetles (Joop et al., 2014), we hypothesise that
446 differential changes in stink gland secretions in response to individual immune experiences
447 are likely contributing to niche construction of flour beetles (Gokhale et al., 2017; Joop et al.,
448 2014) by 1) regulating the pathogenic threat experienced by themselves and other
449 conspecifics (Joop et al., 2014) and 2) mediating aggregation or dispersal behaviours of
450 conspecifics (Duehl et al., 2011). The later process prompts the adjacent conspecifics to
451 disperse into new environments, i.e. “relocational niche construction" or “niche choice”.
452 Similar to the changes in the stink gland secretions, we found effects of treatment and
453 sex on the CHC profiles of adult flour beetles. The CHC profiles of male and female beetles
454 differed strongly in their relative composition (Fig. 4a, b). Sexual dimorphisms of CHC
455 profiles are well documented in various invertebrate species (Jallon & David, 1987; Müller &
456 Müller, 2016; Thomas & Simmons, 2008), but have, to our knowledge, not yet been
457 demonstrated for *T. castaneum*. CHCs play an important role for communication between the
458 sexes (Geiselhardt et al., 2009; Howard & Blomquist, 2005; Müller & Müller, 2016; Thomas
459 & Simmons, 2008), and such communication can also be influenced by immune status. For
460 example, in *Tenebrio molitor* immune challenge modulates sexual signals particularly in
461 males by altering their CHC profiles and volatile odours and thus turns them more attractive
462 to females (Nielsen & Holman, 2012; Sadd et al., 2006). This change in chemical profiles
463 might represent a terminal investment in reproduction by maximising mating prior to death
464 (Sadd et al., 2006).

465 Upon immune treatments, the relative composition of CHC profiles of both wounded
466 and immune-primed beetles changed within one day compared to the naïve controls, and these
467 changes were even more pronounced than for secretions (Fig. 4). The higher proportions of
468 shorter methyl-branched alkanes characterized the immune-stimulated beetles, both wounded
469 and primed, whereas higher proportions of *n*-alkanes were characteristic for the naive beetles
470 (Fig. 4d, f). Interestingly, we found similar results in our previous transcriptome study
471 (Behrens et al. 2014; Table S3) where we observed an upregulation of multiple candidate fatty
472 acid synthase (FAS) genes, 6 h and 18 h post wounding in *T. castaneum* larvae. FASs are
473 involved in the production of methyl alkanes (Blomquist & Ginzel, 2021; Holze et al., 2021).
474 In comparison to *n*-alkanes (linear alkanes), methyl alkanes were shown to be more important
475 in cue recognition (reviewed in van Zweden & d'Ettorre, 2010) and to mediate detection of *T.*
476 *castaneum* larvae by parasitoids (Awater-Salendo et al., 2020). The regulation of methyl
477 alkanes upon immune stimulation has been reported in other host-parasite systems as well
478 (Baracchi et al., 2012; Richard et al., 2008). The greater metabolic cost associated with
479 producing these compounds over *n*-alkanes (Nelson, 1993), along with the variability in the
480 position of methyl branching suggest that methyl alkanes may have an adaptive value in host-
481 parasite interactions. It is also known that many of the CHCs possess antimicrobial properties
482 (Pedrini et al.,2013). The synthesis of insect CHCs and the assimilation/alteration of these
483 compounds during infection by entomopathogenic fungi such as *Beauveria bassiana* may
484 represent an example of a co-evolutionary arms race (Pedrini et al., 2013).
485 Although differences in the relative CHC profiles between the wounded and primed
486 beetles were statistically marginal, the NMDS plots suggested a tendency for the CHC
487 bouquets of wounded beetles, both males and females, to be more strongly separated from
488 those of the naïve beetles than those of the primed group (Fig. 4a, c, e). This is in line with
489 our previous observations that wounding is more effective than priming in eliciting responses
490 among interacting conspecifics (Peuß et al., 2015 and unpublished data). In contrast, Richard

et al. (2008) and Hernández Lopez et al. (2017) found that injection of bacterial lipopolysaccharides (LPS) into honey bee workers (comparable to immune priming) induced markedly different changes in CHCs than wounding, and there was also a tendency for LPS-injections to lead to stronger antimicrobial peptide gene expression changes in queens that had interacted with the treated workers. Most likely, wounding triggers flour beetles to establish a physiological state of heightened 'alertness' that leads to stronger changes in CHC profiles, whereas immune priming carries more information and induces additional physiological and immunological responses that mediate more tailored defences against certain types of pathogens, i.e., specific responses (Ferro et al., 2019; Roth et al., 2009). Taken together, our study suggests that the changed CHC bouquet of wounded beetles is a potential cue for the detection of immune experience in conspecifics, which may lead to the described changes in immune status and *Hsp83*/*Hsp90* expression (Peuß et al., 2015). However, additional experimental tests are needed to prove this.

6. Conclusions

Our study highlights the potential role of chemical profiles that may mediate the intertwined processes of immunity and niche construction in the red flour beetle (Müller et al. 2020). While both stink gland secretions and CHCs are potentially important infochemicals, they might serve different functions. The effects of immune stimulation on secretions were mostly seen in females, whereas the composition of CHCs was affected in both sexes. This supports the view that CHCs might carry information of individual immune experience into the group. In the red flour beetle, the social transfer of such information even has the potential to mediate adaptation via evolutionary capacitance (Peuß et al., 2015). More generally, these results not only demand for the exploration of similar phenomena in related systems with 'external immunity', including other important pest organisms such as the Red Palm Weevil, *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) (Pu et al., 2020), but moreover bear fundamental relevance for questions of disease control in groups and societies.

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Author contributions

JK and CM conceived the ideas. JK, CM, LJT, LKL and RR planned the experimental procedure. LKL and RR carried out the experiments and analysed the raw data together with LJT. BM and LJT carried out the statistical analyses. LKL, RR, LJT, BM and JK wrote the first draft of the manuscript and all authors contributed to the article and approved the submitted version.

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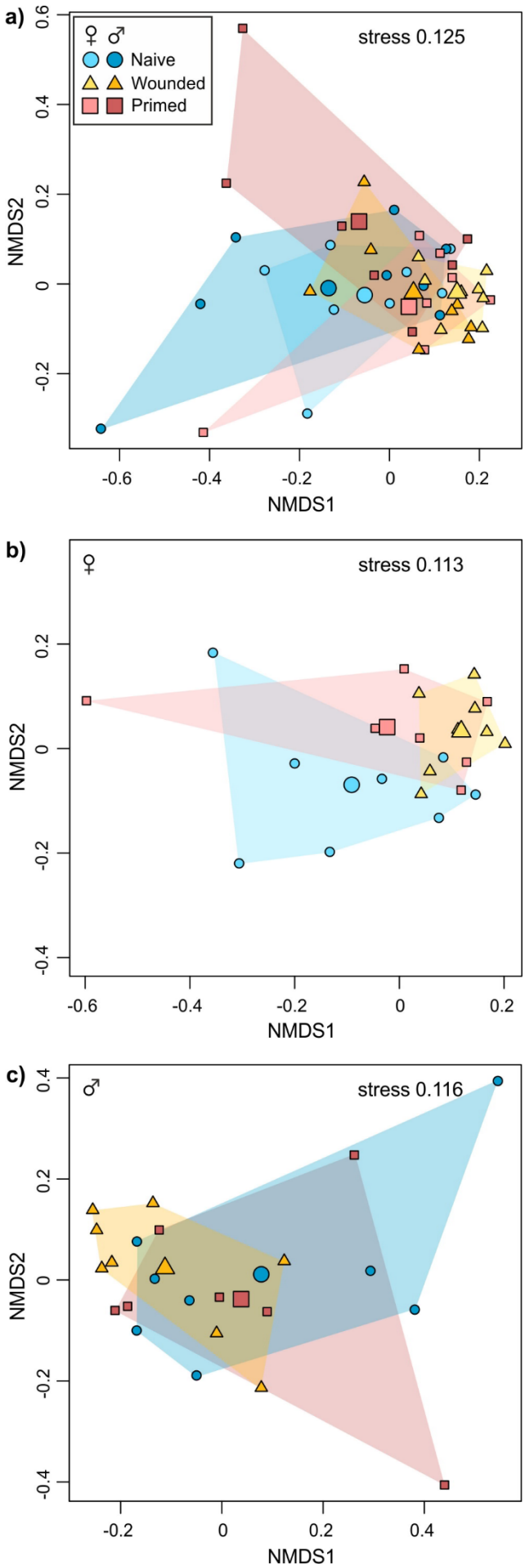
Figures

Fig. 1 Non-metric multidimensional scaling plots of glandular secretion profiles from *Tribolium castaneum* beetles of different immune treatment groups (n = 7-8 per sex and treatment). Beetles were untreated ('Naïve'), or treated by injection of phosphate buffered saline ('Wounded') or of phosphate buffered saline with heat-killed *Bacillus thuringiensis* bv. *tenebrionis* ('Primed'), 24 h before extraction. Plots show profiles of all beetles (**a**), or those of females (**b**) and males separately (**c**). Datasets comprised relative amounts of 26 chemical features (absolute amounts divided by the total amount of all features considered for a sample) and were transformed with Wisconsin square root double standardisation. Dissimilarities are based on pairwise Kulczynki distances. Data points represent profiles of individual beetles, larger symbols show centroids for treatment groups. The stress value is given at the top right of each plot.



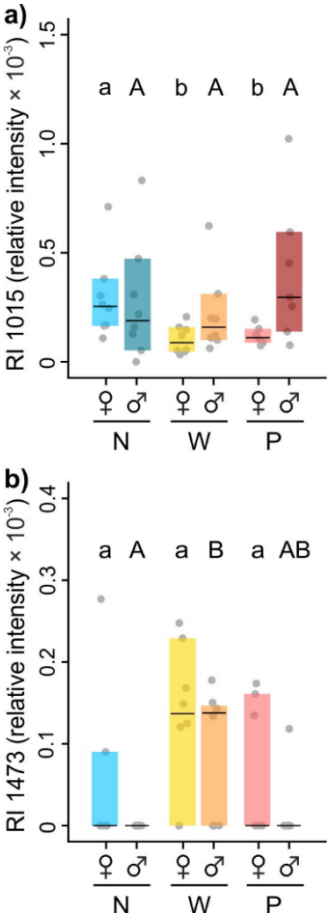


Fig. 2 Relative amounts of two features from glandular secretions of *Tribolium castaneum* beetles of different immune treatment groups (n = 7-8 per sex and treatment), suggested as most important for separation of treatment groups within females (feature RI 1015, **a**) and within males (feature RI 1473, **b**) in random forest analyses. Beetles were untreated (naïve, 'N'), or treated by injection of phosphate buffered saline (wounded, 'W') or of phosphate buffered saline with heat-killed *Bacillus thuringiensis* bv. *tenebrionis* (primed, 'P'), 24 h before extraction. Relative amounts were obtained by dividing absolute amounts by the total amount of all features considered for a sample. Dots represent values of individual beetles, boxes are confidence intervals, lines are medians. Different letters above boxes show significant differences ($p < 0.05$) between treatment groups within females (lower case letters) and males (upper case letters) in Kruskal-Wallis tests after pairwise comparisons.

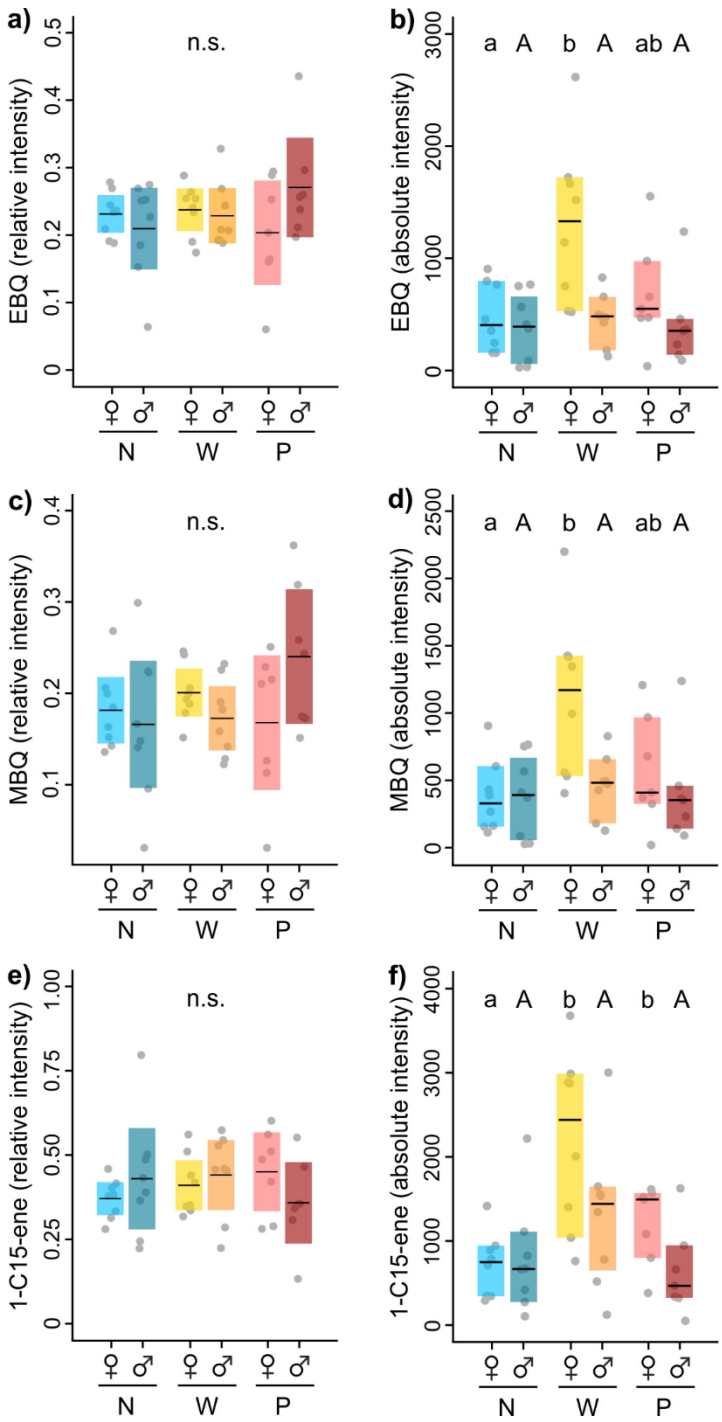


Fig. 3 Relative and absolute amounts of ethyl-1,4-benzoquinone (EBQ), methyl-1,4-benzoquinone (MBQ) and 1-pentadecene (1-C15-ene) from glandular secretions of *Tribolium castaneum* beetles of different immune treatment groups (n = 7-8 per sex and treatment). Beetles were untreated (naïve, 'N'), or treated by injection of phosphate buffered saline (wounded, 'W') or of phosphate buffered saline with heat-killed *Bacillus thuringiensis* bv. *tenebrionis* (primed, 'P'), 24 h before extraction. Relative amounts were obtained by dividing absolute amounts by the total amount of all features considered for a sample. Dots represent values of individual beetles, boxes are confidence intervals, lines are means (**a**-**e**) or medians (**f**). Different letters above boxes show significant differences ($p < 0.05$) between treatment groups within females (lower case letters) and males (upper case letters) in linear models (**a**-**e**) or Kruskal-Wallis tests (**f**) after pairwise comparisons, 'n.s.' is not significant.

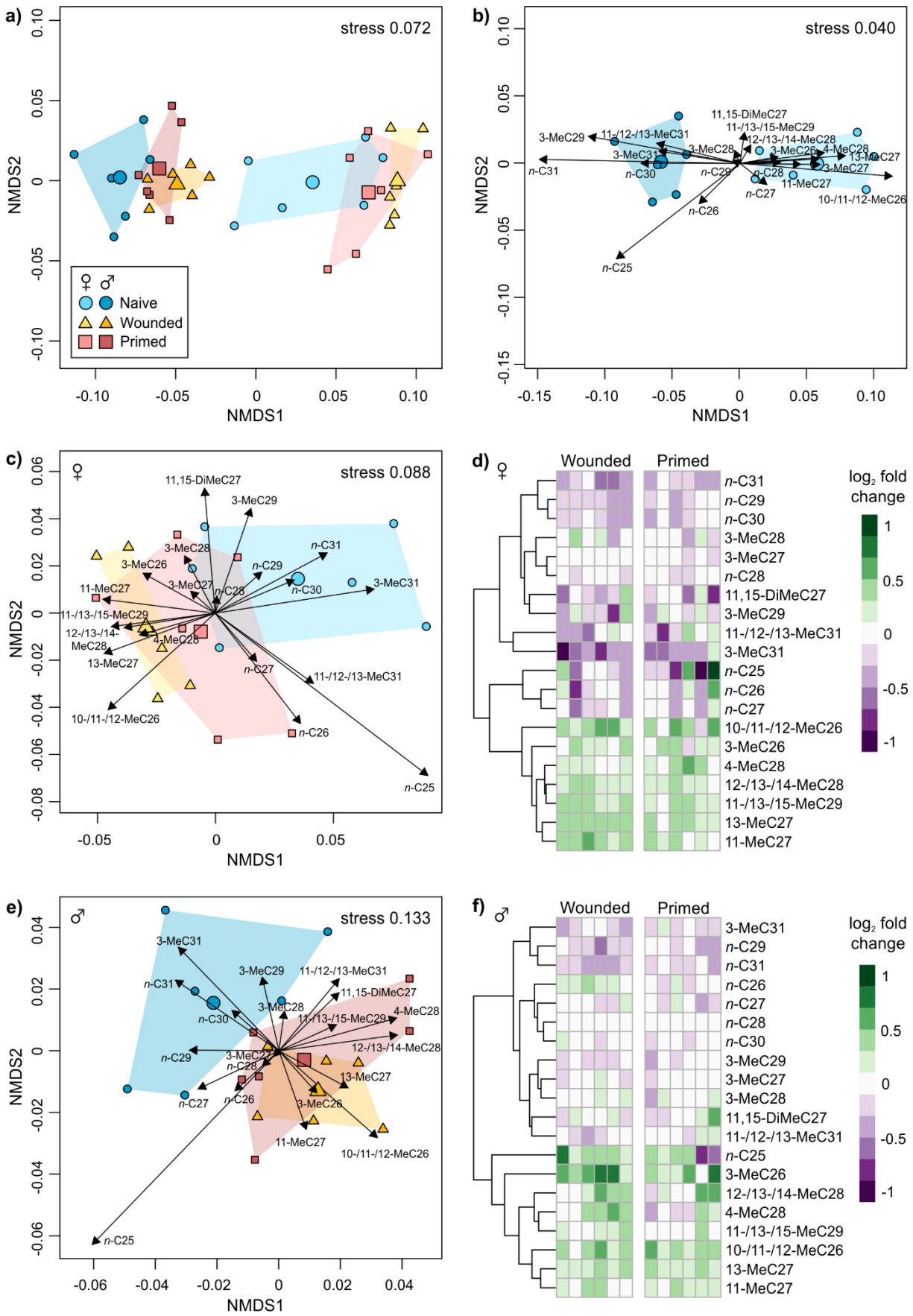


Fig. 4 Non-metric multidimensional scaling (NMDS) plots (**a-c**, **e**) and heatmaps (**d**, **f**) of cuticular hydrocarbon (CHC) profiles from *Tribolium castaneum* beetles of different immune treatment groups (n = 6 per sex and treatment). Beetles were untreated ('Naïve'), or treated by

injection of phosphate buffered saline (‘Wounded’) or of phosphate buffered saline with heat-killed *Bacillus thuringiensis* bv. *tenebrionis* (‘Primed’), 18 h before extraction. All plots are based on relative amounts for individual CHCs (absolute amounts divided by the total amount of all CHCs per sample). NMDS plots show profiles of all beetles (**a**), of naïve beetles separated by sex (**b**), or of females (**c**) and males (**e**) separately. For NMDS plots, datasets comprised 20 CHCs and were transformed with Wisconsin square root double standardisation. Dissimilarities are based on pairwise Kulczynki distances. Data points represent profiles of individual beetles, larger symbols show centroids for treatment groups. The stress value is given at the top right of each plot. Arrows (**b**, **c**, **e**) range from zero to positions of expanded weighted average scores of CHCs. Heatmaps show increase and decrease in the proportion of individual CHCs in wounded and primed females (**d**) and males (**f**) in relation to the median value of female or male naïve beetles as $\log_2$-fold change. Clustering of CHCs is based on Euclidean distances.