Chilling out or heating up? Thermal plasticity of seminal fluid proteins in *Drosophila melanogaster*

Claudia Londoño-Nieto[1], Roberto García-Roa[1,2], Stuart Wigby[3], Irem Sepil [4] and Pau Carazo[1]

[1]Ethology Lab, Cavanilles Institute of Biodiversity and Evolutionary Biology, University of Valencia, Valencia, Spain.

[2] Department of Biology, Lund University, Lund, Sweden.

[3] Department of Evolution, Ecology, and Behaviour, Institute of Infection, Veterinary & Ecological Sciences, University of Liverpool, Liverpool, United Kingdom.

[4] Department of Biology, University of Oxford, Oxford, United Kingdom.

Author for correspondence: Claudia Patricia Londoño Nieto (Claudia.londono@uv.es) +37 674572407.

Abstract

1. Seminal fluid proteins (SFPs) play crucial roles in reproduction by shaping female post-mating physiology and behaviour, male sperm competition, and sexual conflict between the sexes.
2. We mostly ignore how abiotic ecological factors regulate SFP expression and transfer.
3. Here, we use quantitative proteomics in adult flies from a wild *Drosophila melanogaster* population to test how exposure to natural temperature variation (20ºC, 24ºC and 28ºC) across two-time scales (48h and 13d), and under either no or high sperm competition risk, interact to shape SFP production and transfer.
4. We show that both production and transfer of SFPs was reduced after long-term (13d) exposure to low (20ºC) and high (28ºC) temperatures, including in key proteins (sex peptide and ovulin networks) mediating female post-copulatory responses, sperm competition and sexual conflict. Our results show that natural temperature fluctuations can have a strong effect on SFP production and transfer, and thus on post-copulatory sexual selection dynamics.

Keywords: Temperature, ecology, sexual selection, SFPs, reproduction performance, Drosophila, plasticity.

Introduction

The last couple of decades have witnessed a revolution in our understanding of the complexity and function of male ejaculates, much of which has been driven by an increasing realization of the importance of non-sperm components (Gillott, 2003; Perry et al., 2013; Wedell et al., 2002). Sperm is not transferred in isolation within the ejaculate, but alongside a wide variety of molecules, including proteins (seminal fluid proteins: SFPs) that are crucial for reproduction (Hopkins et al., 2017). Produced in the male reproductive organs and transferred to the female during mating, SFPs play essential roles in fertility, sperm storage and usage, and strongly impact the fitness of both sexes by affecting female physiology, behaviour, immunity, and life history, as well as biasing the outcome of sperm competition (Avila et al., 2011; Gillott, 2003; Hopkins & Perry, 2022; Perry et al., 2013; Poiani, 2006). SFPs are thus fundamental to male and female reproductive success and are subject to strong natural and sexual selection (Perry et al., 2013; Sirot et al., 2015). For example, SFPs can alter female physiology and behaviour in ways that are costly to the female, yet beneficial to the male, making them a case study in sexual conflict (Sirot et al., 2015).

Sexual conflict arises because, in sexually reproducing organisms, anisogamy often leads to distinct sex roles (Janicke et al., 2016), which in turn result in divergent reproductive interests between the sexes (Arnqvist & Rowe, 2005; Pizzari & Snook, 2003). Sexual conflict is particularly intense in polygynous species, where it frequently results in male adaptations that increase male fitness at the expense of harming females (i.e. male harm; Arnqvist & Rowe, 2005). Male harming of females is taxonomically widespread and can significantly depress net female productivity, with potentially substantial demographic consequences for populations (Flintham et al., 2023; Gómez-Llano et al., 2024; Martinez-Ruiz & Knell, 2017). An important avenue of male harm are male adaptations driven by strong sperm competition, such as traumatic insemination and “harmful”

53 SFPs (Mueller et al., 2007; Perry et al., 2013; Reinhardt et al., 2015). Work on *Drosophila*
54 *melanogaster* has been at the forefront of this field (Wigby et al., 2020), providing compelling
55 evidence that individual SFPs are sufficient to alter female post-mating physiology and behaviour
56 (Hopkins & Perry, 2022). For example, SFPs such as sex peptide (SP) and ovulin regulate key
57 female post-mating responses. SP stimulates oogenesis, reduces female receptivity to remating,
58 and facilitates sperm storage and release (Chapman, 2001; Chapman et al., 2003; Liu & Kubli,
59 2003; Soller et al., 1999), while ovulin increases the rate of release of existing eggs (ovulation) by
60 the ovaries. Furthermore, other proteins in the SP/ovulin network (i.e., *antr, aqrs, intr, lectin-46Ca,*
61 *lectin-46Cb, Sems, CG17575* and *CG9997*) contribute to the long-term persistence of reduced
62 female receptivity to remating, effective sperm release, and fecundity stimulation (Findlay et al.,
63 2014; Ravi Ram & Wolfner, 2007), while *Semp1* is necessary to process ovulin in mated females
64 (Ravi Ram et al., 2006). Together, SP, ovulin and their associated network proteins increase male
65 reproductive success, and SP and its network proteins are believed to be under sexual conflict, as
66 they increase male fitness but can substantially reduce female lifespan and long-term reproductive
67 output (Wigby & Chapman, 2005).

68 There is increasing evidence that SFPs production and transfer is highly plastic. A male's
69 state, socio-sexual environment and condition can affect both the composition and allocation
70 strategies of his seminal fluid proteome (Wigby et al., 2009). *Drosophila* males, for instance, can
71 tailor the quantity of SFPs they transfer in response to perceived sperm competition risk (Hopkins
72 et al., 2019b), and may even exploit SFPs from rival ejaculates to maximize their own reproductive
73 success (Sirot et al., 2011). There is also evidence that SFPs' post-mating effects on females can
74 be highly plastic themselves (Hopkins & Perry, 2022). However, our understanding of how abiotic
75 ecological factors influence the production and transfer of SFPs, and how these interact with sexual

selection and conflict, remain limited. This gap persists despite mounting evidence suggesting that the environment is a crucial determinant of both fertility and competitive fertilisation success (Sales et al., 2018; Vasudeva et al., 2014; Yun et al., 2017). In particular, responses of SFPs to environmental variables are prime candidates for mediating these effects. For example, studies show that the larval environment can shape SFP allocation patterns later in adult life (Von Hellfeld et al., 2025; Wigby et al., 2016).

Temperature is a particularly salient ecological factor in this respect, due to its potentially far-reaching effects on sexual reproduction and sexual selection/conflict processes (Dougherty et al., 2024; García-Roa et al., 2020). In nature, temperature is subject to substantial spatiotemporal variability at different scales (e.g. circadian, seasonal), potentially imposing strong selective pressures on sperm and ejaculate traits related to sexual selection and reproductive performance, especially in ectothermic species. For instance, high temperatures can impair gonad development, reduce sperm production, viability and function, (Peña et al., 2019; Vasudeva et al., 2014; Wang & Gunderson, 2022), and compromise protein stability and function (Berger et al., 2021). Indeed, *D. melanogaster* SFPs are highly sensitive to heat stress (Canal Domenech & Fricke, 2022), and recent evidence shows that even a small temperature increase, well within the wild females' optimal reproductive range under no sexual conflict (i.e. from 24ºC to 28ºC), is sufficient to reduce both the impact of male ejaculates on female receptivity and overall male harm to females under high sexual conflict (Londoño-Nieto et al., 2023). This finding is especially relevant, as it suggests that even mild, naturally occurring temperature fluctuations may influence SFP production and transfer. Hence, understanding how temperature influences SFP dynamics is crucial to understand how environmental variation shapes male ejaculate composition and sexual selection dynamics, and may also offer important insight into the thermal limits of fertility (Dougherty et al., 2024).

99 Here, we used *D. melanogaster* flies to investigate how natural temperature variation,
100 within the optimal reproductive range for a wild population, affects male production and transfer
101 of SFPs, and how these effects change with perceived sperm competition risk (hereafter SCR). We
102 assessed these effects exposing adult males to temperature (i.e. 20ºC, 24ºC and 28ºC) and SCR
103 treatments (i.e. no SCR vs. high SCR) across two different time scales (experiment 1: short-term
104 exposure-48h; experiment 2: long-term exposure-13d). We predicted that the seminal fluid
105 proteome would be thermally plastic, with 28ºC impacting the production and/or transfer of SFP,
106 particularly after long-term exposure (Berger et al., 2021; Zheng et al., 2024). We also predicted
107 that production/transfer of SFPs under sexual conflict (i.e. the sex peptide, ovulin and their network
108 proteins) would be generally reduced at 28ºC and 20ºC after long-term exposure, based on the fact
109 that male harm is reduced after long-term exposure to 20ºC and 28ºC relative to 24ºC in *Drosophila*
110 *melanogaster* (García-Roa et al., 2019; Londoño-Nieto et al., 2023). More specifically, we
111 predicted that long-term exposure to 28ºC would reduce the abundance of functionally important
112 SFPs involved in mediating female receptivity (i.e. *SP*, *antr, aqrs, intr, lectin-46Ca, lectin-46Cb,*
113 *Sems, CG17575* and *CG9997*), given the reduced impact of male ejaculates on female receptivity
114 after long-term exposure to 28ºC (Londoño-Nieto et al., 2023).

115 **Materials and Methods**

116 Stock Maintenance

117 We carried out all experiments using individuals from our field-collected population, "Vegalibre",
118 established in October 2018 from three wineries in Requena (Spain), and re-sampled annually to
119 maintain natural genetic variation (Londoño-Nieto et al., 2023). We keep Vegalibre in the
120 laboratory with overlapping generations at an average temperature of 24°C with daily pre-
121 programmed fluctuations (±4°C) mimicking natural daily temperature conditions during the

122 reproductively active season, at ~60% humidity and on a 12:12 hr light:dark cycle. We used maize-
123 malt medium (see Londoño-Nieto et al., 2023) as a food source throughout maintenance and
124 experiments. To collect experimental flies, we introduced yeasted grape juice agar plates into stock
125 populations to induce female oviposition. We then collected eggs and placed them in bottles
126 containing ~75 ml of medium to be incubated at 24 ± 4°C at a controlled density (Clancy &
127 Kennington, 2001). We collected virgin flies within 6 hr of eclosion, under ice anesthesia, and then
128 sexed and kept them in vials with food until their use (see below). All females used in our
129 experiments were 4 days old, while males were 4 days old for experiment 1 and 16 days old for
130 experiment 2, at the time when samples were collected.

131 Proteomics experiments

132 *Experimental overview and detailed methods*

133 To study the effect of temperature, and its interaction with perceived SCR, in the production and
134 transfer of SFPs, we exposed adult male flies to either no or high precopulatory levels of SCR
135 (Hopkins et al., 2019b), across three stable temperature treatments of 20°C, 24°C, and 28°C, for
136 two different treatment durations/scales.

137 In experiment 1, we simulated short-term temperature effects (intragenerational effects) on
138 the production and transfer of SFPs. Upon eclosion, we randomly placed virgin males either
139 individually (no-SCR) or in a group of 8 males (high-SCR) in plastic vials with food for 2 days.
140 Next, we randomly divided them into three groups that we allocated to one of the three stable
141 temperature treatments (20°C, 24°C, and 28°C) for 48-hours (Fig. S1).

142 In experiment 2, we examined longer-term temperature effects on the production and
143 transfer of newly synthesized SFPs that would be typical of seasonal (intra- and inter-generational)

144 effects. We took 2 days-old males and first depleted their ejaculates by housing them individually
145 with four virgin females for 24 hours, since *D. melanogaster* males can become sperm and SFP
146 depleted when mated sequentially over a short period (Sirot et al., 2009). Then, we placed the 3
147 days-old depleted males for 13 days in the same temperature-SCR treatments of the experiment 1
148 (Fig. S2). Because males take at least 3 days to fully replenish their SFPs after depletion (Sirot et
149 al., 2009), this design ensured that males synthesized new SFPs under our temperature treatments.

150 *Quantitative proteomics*

151 Immediately after treating males, we explored SFP production patterns at a common garden
152 temperature of 24ºC, by measuring the abundance of SFPs in virgin (experiment 1) or recently
153 unmated (for 13d, experiment 2) males across all temperature-SCR treatment combinations. To
154 explore SFP transfer patterns, we compared the abundance of SFPs in virgin/recently unmated
155 males vs. recently mated males across the same treatment combinations (Hopkins et al., 2019b;
156 Sepil et al., 2019, 2020).

157 Four days before sample collection, we collected females as virgins and held them in same
158 sex groups of 15-20 flies at 24±4°C. On the day of sample collection, we isolated 240 females in
159 vials with food, after which we immediately introduced one focal male either into a vial containing
160 a female or into an empty vial. We flash froze recently mated males in liquid nitrogen 25 min after
161 the start of mating, freezing a virgin/recently unmated male from the same temperature-SCR
162 treatment combination at the same time (Figs. S1-2). Freezing males at 25 min after the start of
163 mating ensures a complete mating (see Hopkins et al., 2019a,b; Sepil et al., 2019). To obtain three
164 independent biological replicates, we repeated this procedure over three different cohorts from the
165 same stock population on different days (i.e. blocks) for each experiment, with all SCR and
166 temperature treatments equally represented within each block.

167 We stored all frozen samples at -80°C until dissection, for which we thawed flash frozen
168 males and dissected their accessory glands, severing the ejaculatory duct and removing the seminal
169 vesicles and testes on ice in phosphate-buffered saline (PBS) under a binocular scope (Hopkins et
170 al., 2019b). Each biological replicate (i.e. sample) consisted of a pool of 20 reproductive glands in
171 25-µL PBS buffer. Samples were analysed with label-free quantitative proteomics, using the
172 sample preparation and quantification protocol (SWATH-MS; Gillet et al., 2012) at the SCISIE
173 proteomics service at the University of Valencia. Samples were centrifuged and protein pellets
174 resuspended in Laemmli buffer. Proteins were separated by SDS-PAGE, full gel separation was
175 performed for spectral library construction, while a short gel run was used to clean and concentrate
176 individual samples, followed by in-gel trypsin digestion as described by Shevchenko et al. (1996).
177 Peptides were analysed using a nanoESI-qQTOF-6600+ TripleTOF mass spectrometer (AB
178 SCIEX) coupled to a nanoflow LC system. Protein identification and quantification were carried
179 out using ProteinPilot and PeakView software with a 1% false discovery rate threshold. Full
180 methodological details are available in SI. We analysed six samples per each temperature
181 treatment-SCR combination (three from virgin or recently unmated males and three from recently
182 mated males). In total, we had 72 samples (36 from experiment 1 and 36 from experiment 2) from
183 1440 males.

184 Data analysis

Scale of inference	Scale at which the factor of interest is applied	Number of replicates at the appropriate scale
Individuals	Sample - consisted of a pool of 20 reproductive glands	6 per each temperature treatment-SCR combination

185

186 We conducted all proteomics analysis on normalized abundances. We normalized protein areas
187 calculated by the total sum of the areas of all quantified proteins. We excluded proteins with fewer
188 than 2 unique peptides to restrict analysis to proteins with high-confidence quantification (Carr et
189 al., 2004). We identified SFPs based on the high-confidence SFPs reference list from Wigby et al.
190 (2020). We used an Elastic net penalized logistic regression model and a partial least-squares
191 discriminant analysis (PLS-DA) to analyse our data sets, using *glmnet* (Friedman et al., 2010),
192 *caret* (Kuhn, 2008) and *mixOmics* (Rohart et al., 2017) packages in RStudio. Elastic net regression
193 is a hybrid method that combines features of Lasso and Ridge regularization (Zou & Hastie, 2005).
194 It is particularly suited for situations where the number of predictors far exceeds the number of
195 observations, as it performs both regularization and variable selection. The method applies a
196 combined Lasso and Ridge penalty that shrinks regression coefficients: weakly associated or
197 irrelevant predictors are penalized to exactly zero, effectively excluding them from the model,
198 whereas variables with stronger effects retain non-zero coefficients. Two parameters control the
199 model: α, which balances the contribution of Lasso (α=1) and Ridge (α=0), and λ, which
200 determines the overall penalty strength. Both parameters were optimized using cross-validation
201 (Zou & Hastie, 2005). Under this framework, proteins with non-zero coefficients represent
202 variables with the strongest effects on proteome quantification.

203 To avoid overparameterization and maximize statistical power, we broke down analyses
204 for both experiments using the following scheme. To analyse how temperature affects SFP
205 production patterns (i.e. the abundance of SFPs in the accessory glands of virgin –in experiment
206 1– or recently unmated –in experiment 2– males), we generated two datasets (one per SCR level),
207 including all virgin (experiment 1) or recently unmated (experiment 2) male samples across all
208 temperature treatments. To analyse how temperature influences SFP allocation, we first inferred

209 the relative abundances of SFPs transferred by dividing the abundance of each SFP in virgin or
210 recently unmated males by its abundance in recently mated males within the same temperature
211 treatment, SCR level, and replicate. Based on these ratios, we then generated two datasets (one per
212 SCR level) including SFP transfer estimates across all temperature treatments. Note, however, that
213 we also analysed absolute abundances of SFPs transferred, with qualitatively very similar results
214 (see SI). To visualize the abundance of SFPs produced and transferred, we took the average across
215 the three biological replicates for each SFP within each temperature treatment and SCR level, and
216 then generated heatmaps using a Euclidean correlation distance metric and plotted with the
217 aheatmap function from the *NMF* package (Gaujoux & Seoighe, 2010) in RStudio (version R 4.2.2;
218 R Core Team, 2022).

219 **Results**

220 We found a total of 1492 proteins, of which we retained 1328 with 2 or more unique peptides.
221 Among these, we focused on the 146 previously identified as SFPs.

222 *Temperature effects on the overall plasticity of SFP production and transfer*

223 Our results show that SFP production and transfer are highly plastic in response to temperature
224 variation across both treatment durations, but with important differences across experiments. In
225 males treated for 48-hour (short-term -experiment 1), elastic net regression identified 74 (under
226 no-SCR) and 3 (under high-SCR) SFPs as predictors contributing most strongly to temperature
227 variation in protein production (see Tables S2 and S3), and 79 (under no-SCR) and 6 (under high-
228 SCR) in the case of SFP transfer (Tables S4 and S5). Heatmaps revealed that both production and
229 transfer of SFPs changed with temperature, with clearer effects under no-SCR. Partial least-squares
230 discriminant analysis (PLS-DA) supported these findings (Fig. 1), suggesting that SFP production

231 and transfer were more strongly affected by temperature in males exposed to no-SCR than in those
232 under high-SCR. Both the number of SFPs showing temperature-mediated changes in abundance
233 and the magnitude of these changes were markedly reduced under high-SCR (Fig. 1), suggesting
234 that under high SCR male ejaculates are less thermally plastic after a short-term exposure. In males
235 treated for 13-days (long-term -experiment 2), elastic net regression selected 39 (under no-SCR)
236 and 68 (under high-SCR) SFPs for production (Tables S6 and S7), and 49 (under no-SCR) and 46
237 (under high-SCR) SFPs for transfer (Tables S8 and S9) as predictors contributing most strongly to
238 differential response to temperature. Heatmap inspection showed clear temperature-driven
239 variation in SFP production and transfer, confirmed by the PLS-DA analysis (Fig. 2). Overall, we
240 found clear evidence that temperature effects after long-term exposure were more uniform across
241 SCR conditions, consistently reducing SPF production and transfer at both low (20ºC) and hot
242 (28ºC) temperatures (Fig. 2). Estimates of SFP transfer derived from absolute abundances
243 produced comparable heatmap patterns, supporting the observed temperature effects (Figs. S3-4).

244 *Temperature effects on the production and transfer of SFPs involved in female post-mating*
245 *responses.*

246 For proteins involved in female post-mating responses, we identified 8 members of the SP network
247 (*antr, aqrs, intr, lectin-46Ca, lectin-46Cb, Sems, CG17575* and *CG9997*) as well as SP itself,
248 ovulin, and its precursor *Semp1*. Our results show that, at 20ºC and 28°C, the production and
249 transfer of these proteins, and specifically those involved in the SP network, frequently diverged
250 from that observed at 24ºC, with clearer effects after the long-term treatment duration (experiment
251 2).

252 In experiment 1 (short-term exposure), SFP production revealed a consistent pattern:
253 protein abundance at 24ºC was markedly reduced compared to 20ºC and 28ºC, but only under no-

254 SCR (Fig. 1A and 3A). Among the selected proteins under no-SCR, SP network proteins *Sems,*
255 *lectin-46Cb* and *CG17575* followed this pattern. In contrast, *SP* decreased and *Semp1* increased at
256 28ºC (Figs. 1A and S5). Under high-SCR we did not identify a consistent pattern. Consistent with
257 differences in production, SFP transfer under no-SCR was generally reduced at 24ºC compared to
258 20ºC and 28ºC, while we did not find a clear temperature-dependent pattern under high-SCR (Fig.
259 1D, and 3B). Moreover, *ovulin, SP* and SP network proteins *lectin-46Cb* and *CG9997* were among
260 those selected as important only under no-SCR (Figs. 1D and S6). Note, however, that the absolute
261 transfer of *SP* was higher at 24ºC than at 20º and 28ºC (Fig. S3). Under high-SCR, *lectin-46Ca*, a
262 SP-network protein, was the only SPF known to regulate female post-mating physiology selected
263 as important, with higher relative and absolute transfer at 28ºC compared to lower temperatures
264 (Figs. 1D. S3 and S6).

265 In experiment 2 (long-term exposure), SFP production showed a clear trend of increased
266 production at 24ºC compared to 20ºC and 28ºC, regardless of SCR (Fig. 2A and 3C). SP and ovulin
267 network proteins (i.e., *Semp1*, *antr*, *SP*, *CG17575)* were only selected as important predictors in
268 males under high-SCR (Fig 2A), showing the general pattern of increased production at 24ºC (Figs
269 2A and S7). *Acp26Ab, Acp53Ea* and *Mst57Dc* were also selected as important proteins only in
270 males under high-SCR but with increased production at 28ºC compared with at lower temperatures
271 (Figs 2A and S7). Patterns of SFP transfer mirrored those observed for production. In both SCR
272 conditions, 24ºC yielded the highest relative and absolute transfer levels (Figs. 2D, 3D and S4).
273 Among proteins showing temperature-dependent transfer, elastic net identified *Semp1*, *antr*,
274 *CG17575, lectin-46Cb, Ovulin* and *Mst57Dc* in males under no-SCR, and *Semp1*, *CG17575,*
275 *lectin-46Cb, lectin-46Ca, Acp53Ea* and *Mst57Dc* in males under high-SCR as those most strongly
276 associated with temperature variation. These proteins exhibited markedly higher transfer at 24ºC

compared to 20ºC and 28ºC (Figs. 2D, S4 and S8). Despite the general similarity across SCR levels, more proteins from the SP and ovulin networks were identified by elastic net as strongly temperature-sensitive in terms of production under high-SCR, while transfer patterns showed similar thermal response across SCR levels (Figs. 2A and 2D).

Discussion

In this study, we show that temperature variation, within the optimal reproductive range for a wild *D. melanogaster* population, affects both the production and transfer of SFPs, with effects shaped by SCR and treatment duration. First, our results show that SFP production and transfer are very plastic in response to temperature. In particular, long-term (13d) exposure to both low (20ºC) and high (28ºC) temperatures resulted in lower production and transfer of SFPs. Second, we show that these effects carry over to proteins with known important functions in female post-mating responses, whereby long-term exposure to high and low temperatures reduced the production and transfer of SFPs involved in the SP and ovulin networks. Overall, these findings highlight the role of natural temperature fluctuations in shaping ejaculate composition, with potential implications for the dynamics of sexual selection, sexual conflict, and temperature effects on reproduction at large.

SFP production and transfer are thermally plastic

In line with our predictions, we found a pronounced plastic response in SFP production and transfer after long-term exposure to high but also to low temperatures, even though both temperatures fall well within the optimal reproductive range for this population in the wild (Londoño-Nieto et al., 2023). Males kept at 20°C or 28°C produced and transferred lower abundances of SFPs than at 24°C across SCR levels. In the long-term exposure experiment, sexually mature males were

exposed to different stable temperatures for 13-days following partial depletion (males can mate consecutively with up to 10 females; Sanghvi et al., 2025) of accessory-glands via repeated matings (Sirot et al., 2009). Thus, our design ensures that males were forced to synthesize new SFPs under our temperature treatments, fulfilling our aim of capturing effects on the production and transfer of new SFPs that would be typical of seasonal effects. Wild *D. melanogaster* flies are likely to live long enough to experience seasonal thermal fluctuations, given that they can survive up to 90 days in controlled conditions (Brischigliaro et al., 2023). Our results thus support the idea that seasonal changes in temperature may impose physiological constraints on the synthesis and/or transfer of SFPs, and fit well with the finding that male-induced harm to females is significantly reduced at 20ºC and 28ºC (vs. 24ºC) in this same population of flies (Londoño-Nieto et al., 2023).

In contrast, our results following short-term exposure to high and low temperatures were less clear and somewhat paradoxical. We again found clear temperature effects on both ejaculate production and transfer, but thermal plasticity was strongly modulated by sociosexual context in a complex way. Under no SCR, we found higher production and transfer at both low and high temperatures, but these differences disappeared under high SCR. Our short-term experiment was designed to capture effects on a fine-grained ecological scale (i.e. short intragenerational effects), given that sexually mature males were exposed to different stable temperatures for 48h and flies likely live considerably longer lifespans in the wild. Compared to our long-term treatment, we may thus expect temperature effects on the production of SFPs to be relatively limited in this treatment.

Broadly, our findings align with previous work showing that high and low temperatures influence several stages of protein synthesis, including transcription, translation, folding, and post-translational modification, which collectively affect protein functionality (Feller & Gerday, 2003; Zheng et al., 2024). In particular, high temperatures are known to be especially detrimental to

322 protein and sperm phenotype and function across animal taxa (Dougherty et al., 2024; Reinhardt
323 et al., 2015; Sales et al., 2018). For instance, heat stress commonly reduces sperm production,
324 motility, viability, and longevity (Wang & Gunderson, 2022). It also increases molecular entropy,
325 which can interfere with correct protein folding and decreases the proportion of functional proteins
326 (Berger et al., 2021). Recent work has extended these findings to seminal fluid components,
327 suggesting that SFPs may also be thermally sensitive (Canal Domenech & Fricke, 2022; Martinet
328 et al., 2023). Our findings build on this work by showing that non-stressful thermal variation within
329 the optimal reproductive range can significantly alter SFP production and transfer. The finding that
330 even subtle and relatively fine-grained temperature variation can have clear effects on SFP
331 production and transfer opens up a new avenue to understand how ecology affects sexual selection,
332 and may also shed light on expected effects of climate change on populations. There is growing
333 recognition that fertility limits, rather than lethal temperature limits, may be more accurate
334 predictors of extinction vulnerability (Dougherty et al., 2024). For instance, some *Drosophila*
335 species can become sterile at temperatures well below their lethal limit (Parratt et al., 2021; Van
336 Heerwaarden & Sgrò, 2021). Our results suggest that, even below these fertility thresholds, natural
337 temperature fluctuations may still influence sexual selection dynamics, potentially increasing
338 vulnerability to climate change in ways not yet fully appreciated (García-Roa et al., 2020).

339 *Long-term exposure to low and high temperatures reduces the production and transfer of SFP that*
340 *mediate key post-mating processes*

341 Beyond the general effects of temperature on SFPs plasticity, we predicted that prolonged exposure
342 to 28ºC would particularly impact functionally important SFPs involved in mediating female
343 receptivity (Londoño-Nieto et al., 2023). Specifically, Londoño-Nieto et al. (2023) showed that
344 ejaculates from males exposed during 13 days to 28°C induced a markedly weaker reduction in

345 female receptivity (i.e. shorter remating latency) compared to males kept at 20ºC and, especially,
346 at 24 °C. Our results fit well with this finding, as, after 13 days of exposure, we observed
347 consistently lower production and transfer of SP -and ovulin- associated proteins at both 28ºC and
348 20ºC compared to 24ºC (Fig 2B).

349 Ejaculate composition is shaped by trade-offs between ejaculate components that mediate
350 post-mating sexual interactions and sperm competition (Perry et al., 2013). Our results thus beg
351 the question of whether temperature may affect males´ ability to strategically adjust the production
352 and transfer of SFPs in response to perceived SCR, where higher investment is typically observed
353 under high-SCR vs. no-SCR conditions (Hopkins et al., 2019b; Wigby et al., 2009). Although our
354 design was not optimized to test this interaction directly, we explored visual comparisons across
355 temperature treatments to explore potential trends in production and transfer of SFPs by males
356 kept under high-SCR vs. no -SCR. We found a trend in that high temperatures seem to disrupt the
357 expected SCR-related patterns of SFP production and transfer in both experiments, especially in
358 SP and ovulin network proteins (Figs. 4 and 5). At 28ºC, after short-term exposure, males under
359 high-SCR produced relatively less of the proteins belonging to the SP and ovulin networks
360 (including ovulin but excluding SP itself) compared to males under no-SCR, than they did at 24ºC
361 (Fig 4A), and transferred less *lectin-46Ca, lectin-46Cb, CG9997,* and *SP* than those under no-SCR
362 (Fig. 4B). After prolonged thermal exposure at 28ºC, males under high-SCR produced less *Semp1,*
363 *lectin-46Cb, lectin-46Ca, intr, Sems, antr, CG17575* and *SP* (Fig. 5A), and transferred less *Sems,*
364 *lectin-46Ca*, *antr*, *Semp1, ovulin* and *SP* than males under no-SCR, than they did at 24ºC (Fig.
365 5B). This trend must be taken as preliminary, but suggests that high temperatures may limit males'
366 ability to strategically adjust their ejaculate composition in response to SCR.

367 *The ecological relevance of thermal effects on SFPs*

368 Taken together, our results underscore the broader implications of thermal sensitivity in ejaculate
369 composition. Due to the importance of SFPs, their plasticity (or lack thereof) under thermal
370 variation could mediate individual fitness and shape population responses to environmental
371 change. Our results from experiment 1 suggest that short term fine-scale temperature fluctuations
372 (e.g. circadian) are unlikely to have major effects on females and populations, especially under
373 high SCR. However, our results from experiment 2 capture the cumulative impact of thermal
374 variation on ejaculate production and transfer over a timeframe that spans both intra- and inter-
375 generational seasonal effects. Such thermal fluctuations are extremely common in the field
376 (particularly in temperate climates), and thus are of clear ecological relevance.

377 At the intragenerational scale, sustained temperature effects on continuous SFP production
378 and transfer are bound to alter male reproductive dynamics and overall reproductive success across
379 an individual's lifetime, potentially making plasticity itself a target of selection. Supporting this
380 idea, there is evidence showing that seminal fluid composition can vary among genotypes in
381 response to environmental conditions, with direct consequences for reproductive success (Patlar
382 & Ramm, 2020). In addition, *D. melanogaster* from the same population used in this study show
383 strong thermal GxE interactions in male reproductive success (Londoño-Nieto et al., 2025).
384 Interestingly, these GxE effects appear to be sexually dimorphic (Panyam et al., unpublished
385 results), indicating that males and females may experience, and potentially adapt to, thermal
386 variation in different ways. Moreover, a recent experimental evolution experiment shows the
387 thermal environment can drive quick evolutionary changes in SFPs composition (Londoño-Nieto
388 et al., 2025).

389 At the intergenerational scale, prolonged periods of heat or cold may shift the targets of
390 post-copulatory sexual selection across seasons, potentially driving seasonal adaptive tracking

and/or balancing selection (Bergland et al., 2014; Hoffmann et al., 2005; Machado et al., 2021; Rudman et al., 2022). For example, if higher temperatures consistently reduce the abundance or effectiveness of key proteins like SP, this could weaken male ability to manipulate female post-mating behaviour, potentially shifting the relative importance of pre- vs. post-copulatory sexual selection towards the former. As climate change increases the frequency and magnitude of temperature fluctuations, such effects may be increasingly important for temperate populations of ectothermic species.

Conclusions

Our results show that natural temperature fluctuations can have a strong effect on SFP production and transfer, and thus on post-copulatory sexual selection dynamics. Notably, long-term exposure to low and high temperatures consistently reduced the production and transfer of SFPs, including key proteins involved in female post-mating responses. These findings highlight how seasonal environmental variation can shape ejaculate traits under realistic ecological conditions, and provide a mechanistic link for effects shown in recent studies linking habitat complexity, reproductive plasticity, and eco-evolutionary processes shaping sexual selection (Berger & Liljestrand-Rönn, 2024; Gomez-Llano et al., 2018; Londoño-Nieto et al., 2023, 2025; Malek & Long, 2019; Yun et al., 2017, 2018). Importantly, these results may have important implications for understanding species vulnerability to climate change. We suggest a focus of future studies should be to examine how temperature influences the male´s ability to tailor SPF production and transfer in response to SCR, as well as the plasticity in female reproductive traits (i.e. their responses to male ejaculates), and how these female traits co-evolve with male ejaculates in response to temperature variation. Finally, these findings may also have applied relevance, as the thermal sensitivity of SFPs could improve strategies in pest control and assisted reproduction.

References

Arnqvist, G., & Rowe, L. (2005). *Sexual Conflict*. Princeton University Press, Princeton, NJ.

Avila, F. W., Sirot, L. K., LaFlamme, B. A., Rubinstein, C. D., & Wolfner, M. F. (2011). Insect Seminal Fluid Proteins: Identification and Function. *Annual Review of Entomology*, *56*(1), 21–40. https://doi.org/10.1146/annurev-ento-120709-144823

Berger, D., & Liljestrand-Rönn, J. (2024). Environmental complexity mitigates the demographic impact of sexual selection. *Ecology Letters*, *27*(1), e14355. https://doi.org/10.1111/ele.14355

Berger, D., Stångberg, J., Baur, J., & Walters, R. (2021). Elevated temperature increases genome-wide selection on de novo mutations. *Proceedings of the Royal Society B: Biological Sciences*, *287*. https://doi.org/10.1098/rspb.2020.3094

Bergland, A. O., Behrman, E. L., Brien, K. R. O., Schmidt, P. S., & Petrov, D. A. (2014). Genomic Evidence of Rapid and Stable Adaptive Oscillations over Seasonal Time Scales in Drosophila. *PLoS Genetics*, *10*(11). https://doi.org/10.1371/journal.pgen.1004775

Brischigliaro, M., Fernandez-Vizarra, E., & Viscomi, C. (2023). Mitochondrial Neurodegeneration: Lessons from Drosophila melanogaster Models. *Biomolecules*, *13*(2), 378. https://doi.org/10.3390/biom13020378

Canal Domenech, B., & Fricke, C. (2022). Recovery from heat-induced infertility—A study of reproductive tissue responses and fitness consequences in male *Drosophila melanogaster*. *Ecology and Evolution*, *12*(12), e9563. https://doi.org/10.1002/ece3.9563

Carr, S., Aebersold, R., Baldwin, M., Burlingame, A., Clauser, K., & Nesvizhskii, A. (2004). The Need for Guidelines in Publication of Peptide and Protein Identification Data. *Molecular & Cellular Proteomics*, *3*(6), 531–533. https://doi.org/10.1074/mcp.T400006-MCP200

Chapman, T. (2001). Seminal fluid-mediated fitness traits in Drosophila. *Genetics*, *87*.

Chapman, T., Bangham, J., Vinti, G., Seifried, B., Lung, O., Wolfner, M. F., Smith, H. K., & Partridge, L. (2003). The sex peptide of Drosophila melanogaster: Female post-mating responses analyzed by using RNA interference. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(17), 9923–9928. https://doi.org/10.1073/pnas.1631635100

Clancy, D. J., & Kennington, J. (2001). A simple method to achieve consistent larval density in bottle cultures. *Drosophila Information Service*, *84*(January), 168–169.

Dougherty, L. R., Frost, F., Maenpaa, M. I., Rowe, M., Cole, B. J., Vasudeva, R., Pottier, P., Schultner, E., Macartney, E. L., Lindenbaum, I., Smith, J. L., Carazo, P., Graziano, M., Weaving, H., Domenech, B. C., Berger, D., Meena, A., Bishop, T. R., Noble, D. W. A., … Price, T. A. R. (2024). A systematic map of studies testing the relationship between temperature and animal reproduction. *Ecological Solutions and Evidence*, *5*(1). https://doi.org/DOI:%252010.1002/2688-8319.12303

Feller, G., & Gerday, C. (2003). Psychrophilic enzymes: Hot topics in cold adaptation. *Nature Reviews Microbiology*, *1*(3), 200–208. https://doi.org/10.1038/nrmicro773

Findlay, G. D., Sitnik, J. L., Wang, W., Aquadro, C. F., Clark, N. L., & Wolfner, M. F. (2014). Evolutionary Rate Covariation Identifies New Members of a Protein Network Required for Drosophila melanogaster Female Post-Mating Responses. *PLoS Genetics*, *10*(1), e1004108. https://doi.org/10.1371/journal.pgen.1004108

Flintham, E. O., Savolainen, V., & Mullon, C. (2023). Male harm offsets the demographic benefits of good genes. *Proceedings of the National Academy of Sciences of the United States of America*, *120*(10), 1–9. https://doi.org/10.1073/pnas.2211668120

Friedman, J., Hastie, T., & Tibshirani, R. (2010). Regularization Paths for Generalized Linear Models via Coordinate Descent. *Journal of Statistical Software*, *33*(1). https://doi.org/10.18637/jss.v033.i01

García-Roa, R., Chirinos, V., & Carazo, P. (2019). The ecology of sexual conflict: Temperature variation in the social environment can drastically modulate male harm to females. *Functional Ecology*, *33*(4), 681–692. https://doi.org/10.1111/1365-2435.13275

García-Roa, R., Garcia-Gonzalez, F., Noble, D. W. A., & Carazo, P. (2020). Temperature as a modulator of sexual selection. *Biological Reviews*, *95*(6), 1607–1629. https://doi.org/10.1111/brv.12632

Gaujoux, R., & Seoighe, C. (2010). A flexible R package for nonnegative matrix factorization. *BMC Bioinformatics*, *11*. https://doi.org/10.1186/1471-2105-11-367

Gillet, L. C., Navarro, P., Tate, S., Röst, H., Selevsek, N., Reiter, L., Bonner, R., & Aebersold, R. (2012). Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: A new concept for consistent and accurate proteome analysis. *Molecular and Cellular Proteomics*, *11*(6), 1–17. https://doi.org/10.1074/mcp.O111.016717

Gillott, C. (2003). Male Accessory Gland Secretions: Modulators of Female Reproductive Physiology and Behavior. *Annual Review of Entomology*, *48*(1), 163–184. https://doi.org/10.1146/annurev.ento.48.091801.112657

Gomez-Llano, M. A., Bensch, H. M., & Svensson, E. I. (2018). Sexual conflict and ecology: Species composition and male density interact to reduce male mating harassment and increase female survival. *Evolution*, *72*(4), 906–915. https://doi.org/10.1111/evo.13457

Gómez-Llano, M., Faria, G. S., García-Roa, R., Noble, D. W. A., & Carazo, P. (2024). Male harm suppresses female fitness, affecting the dynamics of adaptation and evolutionary rescue. *Evolution Letters*, *8*(1), 149–160. https://doi.org/10.1093/evlett/qrac002

Hoffmann, A. A., Hallas, R., Anderson, A., & Telonis-Scott, M. (2005). *Evidence for a robust sex-specific trade-off between cold resistance and starvation resistance in Drosophila melanogaster*. *18*, 804–810. https://doi.org/10.1111/j.1420-9101.2004.00871.x

Hopkins, B. R., & Perry, J. C. (2022). The evolution of sex peptide: Sexual conflict, cooperation, and coevolution. *Biological Reviews*, *97*(4), 1426–1448. https://doi.org/10.1111/brv.12849

Hopkins, B. R., Sepil, I., Bonham, S., Miller, T., Charles, P. D., Fischer, R., Kessler, B. M., Wilson, C., & Wigby, S. (2019a). BMP signaling inhibition in Drosophila secondary cells remodels the seminal proteome and self and rival ejaculate functions. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(49), 24719–24728. https://doi.org/10.1073/pnas.1914491116

Hopkins, B. R., Sepil, I., Thézénas, M. L., Craig, J. F., Miller, T., Charles, P. D., Fischer, R., Kessler, B. M., Bretman, A., Pizzari, T., & Wigby, S. (2019b). Divergent allocation of sperm and the seminal proteome along a competition gradient in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(36), 17925–17933. https://doi.org/10.1073/pnas.1906149116

Hopkins, B. R., Sepil, I., & Wigby, S. (2017). Seminal fluid. *Current Biology*, *27*(11), R404–R405. https://doi.org/10.1016/j.cub.2017.03.063

Janicke, T., Häderer, I. K., Lajeunesse, M. J., & Anthes, N. (2016). Evolutionary Biology: Darwinian sex roles confirmed across the animal kingdom. *Science Advances*, *2*(2), 1–11. https://doi.org/10.1126/sciadv.1500983

Kuhn, M. (2008). Building Predictive Models in *R* Using the **caret** Package. *Journal of Statistical Software*, *28*(5). https://doi.org/10.18637/jss.v028.i05

LaFlamme, B. A., Avila, F. W., Michalski, K., & Wolfner, M. F. (2014). A *Drosophila* Protease Cascade Member, Seminal Metalloprotease-1, Is Activated Stepwise by Male Factors and Requires Female Factors for Full Activity. *Genetics*, *196*(4), 1117–1129. https://doi.org/10.1534/genetics.113.160101

Liu, H., & Kubli, E. (2003). Sex-peptide is the molecular basis of the sperm effect in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(17), 9929–9933. https://doi.org/10.1073/pnas.1631700100

Londoño-Nieto, C., Butler-Margalef, M., García-Roa, R., & Carazo, P. (2025). Temperature Drives the Evolutionary Diversification of Male Harm in *Drosophila melanogaster* Flies. *Ecology Letters*, *28*(3), e70102. https://doi.org/10.1111/ele.70102

Londoño-Nieto, C., García-Roa, R., Garcia-Co, C., González, P., & Carazo, P. (2023). Thermal phenotypic plasticity of pre- and post-copulatory male harm buffers sexual conflict in wild Drosophila melanogaster. *eLife*, *12*, e84759. https://doi.org/10.7554/eLife.84759

Machado, H. E., Bergland, A. O., Taylor, R., Tilk, S., Behrman, E., Dyer, K., Fabian, D. K., Flatt, T., González, J., Karasov, T. L., Kim, B., Kozeretska, I., Lazzaro, B. P., Merritt, T. J., Pool, J. E., O'Brien, K., Rajpurohit, S., Roy, P. R., Schaeffer, S. W., … Petrov, D. A. (2021). Broad geographic sampling reveals the shared basis and environmental correlates

of seasonal adaptation in Drosophila. *eLife*, *10*, e67577. https://doi.org/10.7554/eLife.67577

Malek, H., & Long, T. (2019). Spatial environmental complexity mediates sexual conflict and sexual selection in Drosophila melanogaster. *Ecology and Evolution*, *9*(5). https://doi.org/10.1002/ece3.4932

Martinet, B., Przybyla, K., Decroo, C., Wattiez, R., & Aron, S. (2023). Proteomic differences in seminal fluid of social insects whose sperm differ in heat tolerance. *Royal Society Open Science*, *10*(11), 231389. https://doi.org/10.1098/rsos.231389

Martinez-Ruiz, C., & Knell, R. J. (2017). Sexual selection can both increase and decrease extinction probability: Reconciling demographic and evolutionary factors. *Journal of Animal Ecology*, *86*, 117–127. https://doi.org/doi:%252010.1111/1365-2656.12601

Mueller, J. L., Page, J. L., & Wolfner, M. F. (2007). An Ectopic Expression Screen Reveals the Protective and Toxic Effects of Drosophila Seminal Fluid Proteins. *Genetics*, *175*(2), 777–783. https://doi.org/10.1534/genetics.106.065318

Parratt, S. R., Walsh, B. S., Metelmann, S., White, N., Manser, A., Bretman, A. J., Hoffmann, A. A., Snook, R. R., & Price, T. A. R. (2021). Temperatures that sterilize males better match global species distributions than lethal temperatures. *Nature Climate Change*, *11*(6), 481–484. https://doi.org/10.1038/s41558-021-01047-0

Patlar, B., & Ramm, S. A. (2020). Genotype-by-environment interactions for seminal fluid expression and sperm competitive ability. *Journal of Evolutionary Biology*, *33*(2), 225–236. https://doi.org/10.1111/jeb.13568

Peña Jr., S. T., Stone, F., Gummow, B., Parker, A. J., & Paris, D. B. B. P. (2019). Tropical summer induces DNA fragmentation in boar spermatozoa: Implications for evaluating

seasonal infertility. *Reproduction, Fertility and Development*, *31*(3), 590. https://doi.org/10.1071/RD18159

Perry, J. C., Sirot, L., & Wigby, S. (2013). The seminal symphony: How to compose an ejaculate. *Trends in Ecology and Evolution*, *28*(7), 414–422. https://doi.org/10.1016/j.tree.2013.03.005

Pizzari, T., & Snook, R. R. (2003). Perspective: Sexual conflict and sexual selection: Chasing away paradigm shifts. *Evolution*, *57*(6), 1223–1236. https://doi.org/10.1111/j.0014-3820.2003.tb00331.x

Poiani, A. (2006). Complexity of seminal fluid: A review. *Behavioral Ecology and Sociobiology*, *60*(3), 289–310. https://doi.org/10.1007/s00265-006-0178-0

R Core Team. (2022). *R: A language and environment for statistical computing.* [Computer software]. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Ravi Ram, K., Sirot, L. K., & Wolfner, M. F. (2006). Predicted seminal astacin-like protease is required for processing of reproductive proteins in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences*, *103*(49), 18674–18679. https://doi.org/10.1073/pnas.0606228103

Ravi Ram, K., & Wolfner, M. F. (2007). Seminal influences: Drosophila Acps and the molecular interplay between males and females during reproduction. *Integrative and Comparative Biology*, *47*(3), 427–445. https://doi.org/10.1093/icb/icm046

Reinhardt, K., Anthes, N., & Lange, R. (2015). Copulatory wounding and traumatic insemination. *Cold Spring Harbor Perspectives in Biology*, *7*(5), 1–26. https://doi.org/10.1101/cshperspect.a017582

Rohart, F., Gautier, B., Singh, A., & Lê Cao, K.-A. (2017). mixOmics: An R package for ‘omics feature selection and multiple data integration. *PLOS Computational Biology*, *13*(11), e1005752. https://doi.org/10.1371/journal.pcbi.1005752

Rudman, S. M., Greenblum, S. I., Rajpurohit, S., Betancourt, N. J., Hanna, J., Tilk, S., Yokoyama, T., Petrov, D. A., & Schmidt, P. (2022). Direct observation of adaptive tracking on ecological time scales in *Drosophila*. *Science*, *375*(6586), eabj7484. https://doi.org/10.1126/science.abj7484

Sales, K., Vasudeva, R., Dickinson, M. E., Godwin, J. L., Lumley, A. J., Michalczyk, Ł., Hebberecht, L., Thomas, P., Franco, A., & Gage, M. J. G. (2018). Experimental heatwaves compromise sperm function and cause transgenerational damage in a model insect. *Nature Communications*, *9*(1), 4771. https://doi.org/10.1038/s41467-018-07273-z

Sanghvi, K., Shandilya, S., Brown, A., Todorova, B., Jahn, M., Gascoigne, S. J. L., Camilleri, T.-L., Pizzari, T., & Sepil, I. (2025). Reproductive output of old males is limited by seminal fluid, not sperm number. *Evolution Letters*, *9*(2), 282–291. https://doi.org/10.1093/evlett/qrae071

Sepil, I., Hopkins, B. R., Dean, R., Bath, E., Friedman, S., Swanson, B., Ostridge, H. J., Harper, L., Buehner, N. A., Wolfner, M. F., Konietzny, R., Thézénas, M.-L., Sandham, E., Charles, P. D., Fischer, R., Steinhauer, J., Kessler, B. M., & Wigby, S. (2020). Male reproductive aging arises via multifaceted mating-dependent sperm and seminal proteome declines, but is postponable in *Drosophila*. *Proceedings of the National Academy of Sciences*, *117*(29), 17094–17103. https://doi.org/10.1073/pnas.2009053117

Sepil, I., Hopkins, B. R., Dean, R., Thézénas, M.-L., Charles, P. D., Konietzny, R., Fischer, R., Kessler, B. M., & Wigby, S. (2019). Quantitative Proteomics Identification of Seminal

Fluid Proteins in Male Drosophila melanogaster. *Molecular & Cellular Proteomics*, *18*, S46–S58. https://doi.org/10.1074/mcp.RA118.000831

Shevchenko, A., Jensen, O. N., Podtelejnikov, A. V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenko, A., Boucherie, H., & Mann, M. (1996). Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(25), 14440–14445. https://doi.org/10.1073/pnas.93.25.14440

Sirot, L. K., Buehner, N. A., Fiumera, A. C., & Wolfner, M. F. (2009). Seminal fluid protein depletion and replenishment in the fruit fly, Drosophila melanogaster: An ELISA-based method for tracking individual ejaculates. *Behavioral Ecology and Sociobiology*, *63*(10), 1505–1513. https://doi.org/10.1007/s00265-009-0806-6

Sirot, L. K., Wolfner, M. F., & Wigby, S. (2011). Protein-specific manipulation of ejaculate composition in response to female mating status in Drosophila melanogaster. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(24), 9922–9926. https://doi.org/10.1073/pnas.1100905108

Sirot, L. K., Wong, A., Chapman, T., & Wolfner, M. F. (2015). Sexual Conflict and Seminal Fluid Proteins: A Dynamic Landscape of Sexual Interactions. *Cold Spring Harbor Perspectives in Biology*, *7*(2), a017533. https://doi.org/10.1101/cshperspect.a017533

Soller, M., Bownes, M., & Kubli, E. (1999). Control of Oocyte Maturation in Sexually MatureDrosophilaFemales. *Developmental Biology*, *208*(2), 337–351. https://doi.org/10.1006/dbio.1999.9210

Van Heerwaarden, B., & Sgrò, C. M. (2021). Male fertility thermal limits predict vulnerability to climate warming. *Nature Communications*, *12*(1), 2214. https://doi.org/10.1038/s41467-021-22546-w

Vasudeva, R., Deeming, D. C., & Eady, P. E. (2014). Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition in *Callosobruchus maculatus*. *Journal of Evolutionary Biology*, *27*(9), 1811–1818. https://doi.org/10.1111/jeb.12431

Von Hellfeld, R., Konietzny, R., Charles, P. D., Fischer, R., Kessler, B. M., Wigby, S., Sepil, I., & Morimoto, J. (2025). *The developmental environment mediates adult seminal proteome allocation in male* Drosophila melanogaster. Evolutionary Biology. https://doi.org/10.1101/2025.01.09.632097

Wang, W. W.-Y., & Gunderson, A. R. (2022). The Physiological and Evolutionary Ecology of Sperm Thermal Performance. *Frontiers in Physiology*, *13*, 754830. https://doi.org/10.3389/fphys.2022.754830

Wedell, N., Gage, M. J. G., & Parker, G. A. (2002). Sperm competition, male prudence and sperm-limited females. *TREE*, *17*(7), 313–320.

Wigby, S., Brown, N. C., Allen, S. E., Misra, S., Sitnik, J. L., Sepil, I., Clark, A. G., & Wolfner, M. F. (2020). The Drosophila seminal proteome and its role in postcopulatory sexual selection. *Philosophical Transactions of the Royal Society of London B Biological Sciences*, 20200072.

Wigby, S., & Chapman, T. (2005). Sex peptide causes mating costs in female Drosophila melanogaster. *Current Biology*, *15*(4), 316–321.

Wigby, S., Perry, J. C., Kim, Y., & Sirot, L. K. (2016). Developmental environment mediates male seminal protein investment in *D rosophila melanogaster* . *Functional Ecology*, *30*(3), 410–419. https://doi.org/10.1111/1365-2435.12515

Wigby, S., Sirot, L. K., Linklater, J. R., Buehner, N., Calboli, F. C. F., Bretman, A., Wolfner, M. F., & Chapman, T. (2009). Seminal Fluid Protein Allocation and Male Reproductive Success. *Current Biology*, *19*(9), 751–757. https://doi.org/10.1016/j.cub.2009.03.036

Yun, L., Chen, P. J., Kwok, K. E., Angell, C. S., Rundle, H. D., & Agrawal, A. F. (2018). Competition for mates and the improvement of nonsexual fitness. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(26), 6762–6767. https://doi.org/10.1073/pnas.1805435115

Yun, L., Chen, P. J., Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). The physical environment mediates male harm and its effect on selection in females. *Proceedings of the Royal Society B: Biological Sciences*. https://doi.org/10.1098/rspb.2017.0424

Zheng, J., Guo, N., Huang, Y., Guo, X., & Wagner, A. (2024). High temperature delays and low temperature accelerates evolution of a new protein phenotype. *Nature Communications*, *15*(1), 2495. https://doi.org/10.1038/s41467-024-46332-6

Zou, H., & Hastie, T. (2005). Regularization and Variable Selection Via the Elastic Net. *Journal of the Royal Statistical Society Series B: Statistical Methodology*, *67*(2), 301–320. https://doi.org/10.1111/j.1467-9868.2005.00503.x

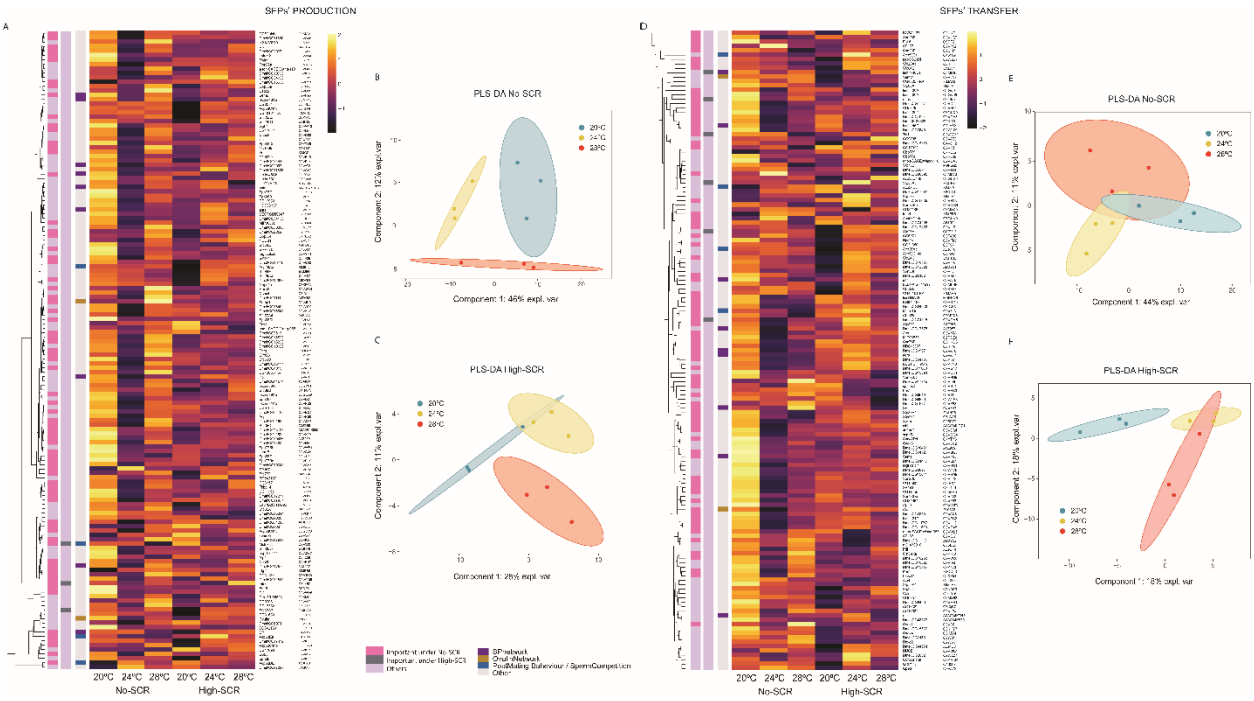


Figure 1 | Effect of temperature on SFPs production and transfer (short-term exposure / experiment 1). A) Heatmap showing the abundance of 145 SFPs detected in accessory gland samples. Elastic net regression selected 74 (under no-SCR) and 3 (under High-SCR) out of 145 SFPs as predictors with a strong effect differentiating proteomic responses across temperature treatments. Each cell gives the across-biological replicate mean for that protein, at each temperature treatment. Row annotations indicate SFPs with strong response to temperature in each SCR level and include functional information relating to protein functions as part of the sex peptide or ovulin networks, or other known roles in sperm competition. B) Partial least-squares discriminant analysis (PLS-DA) plot of proteins production for no-SCR treatment and C) PLS-DA plot of proteins production for high-SCR treatment. Points represent biological replicates. Ellipses denote variability among replicates. D) Heatmap showing the inferred relative abundance of 145 SFPs transferred to females during mating. Elastic net regression selected 79 (no SCR treatment) and 6 (High SCR treatment) out of 145 SFPs as predictors with strong differential transfer response to temperature. Each cell shows the mean ratio of protein abundance in virgin males relative to recently mated males, across biological replicates, for each temperature treatment, reflecting the relative loss of protein upon mating. Values closer to 1 indicate proteins reduced after mating (i.e., transferred). Heatmaps were generated from log2-transformed protein abundances for better visualization. E) PLS- DA plot of proteins transfer for no-SCR treatment and F) PLS-DA plot of proteins transfer for high-SCR treatment. Points in each PLS-DA plot represent biological replicates. Ellipses denote variability among replicates.

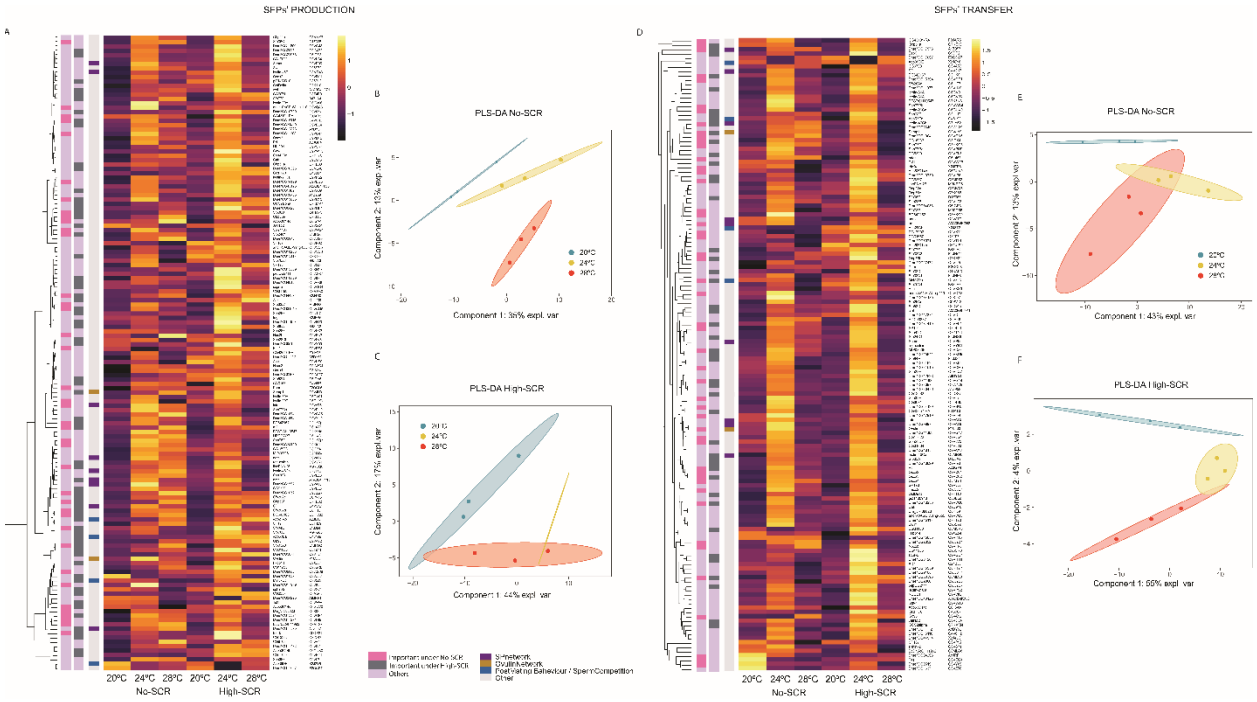


Figure 2 | Effect of temperature on SFPs production and transfer (long-term exposure / experiment 2). A) Heatmap showing the abundance of the 145 SFPs detected in the accessory gland samples. Elastic net regression selected 39 (no SCR treatment) and 68 (High SCR treatment) out of 145 SFPs as predictors with a strong effect differentiating proteomic responses across temperature treatments. Each cell gives the across-biological replicate mean for that protein, at each temperature treatment. Row annotations indicate SFPs with strong response to temperature in each SCR level and include functional information relating to protein functions as part of the sex peptide or ovulin networks, or other known roles in sperm competition. B) PLS-DA plot of proteins production for no-SCR treatment and C) PLS- DA plot of proteins production for high-SCR treatment. Points represent biological replicates. Ellipses denote variability among replicates. D) Heatmap showing the inferred relative abundance of 145 SFPs transferred to females during mating. Elastic net regression selected 49 (no SCR treatment) and 46 (High SCR treatment) out of 145 SFPs as predictors with strong differential transfer response to temperature. Each cell shows the mean ratio of protein abundance in unmated males relative to recently mated males, across biological replicates, for each temperature treatment, reflecting the relative loss of protein upon mating. Values closer to 1 indicate proteins reduced after mating (i.e., transferred). Heatmaps were generated from log2-transformed protein abundances for better visualization. E) PLS- DA plot of proteins transfer for no-SCR treatment and F) PLS-DA plot of proteins transfer for high-SCR treatment. Points in each PLS-DA plot represent biological replicates. Ellipses denote variability among replicates.

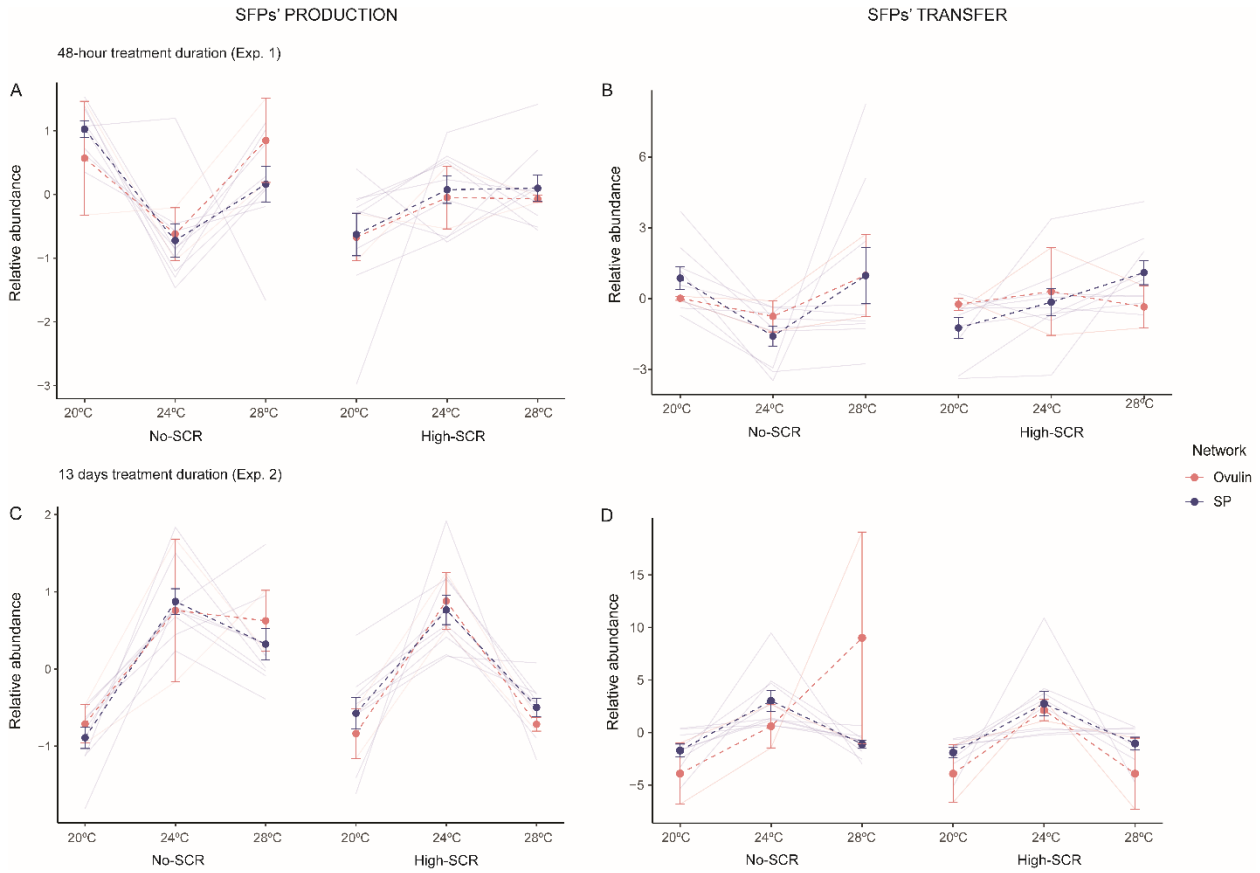


Figure 3 | Effect of temperature on production and transfer of SFPs involved in Ovulin and SP networks. A – B) Relative abundance production and transfer profiles of SPFs associated with the SP and ovulin networks across different temperatures and SCR levels for short-term exposure (experiment 1). C – D) Relative abundance production and transfer profiles of SPFs associated with the SP and ovulin networks across different temperatures and SCR levels for long-term exposure (experiment 2). Each faint line represents the mean across the 3 replicates for an individual SFP within each treatment combination. Abundance values were normalized by mean-centering and averaged across replicates. Blue points represent the average (± s.e.) of SPFs in the SP network, across temperature and SCR conditions. Red points represent the average (± s.e.) of SFPs in the ovulin network, across temperature and SCR conditions.

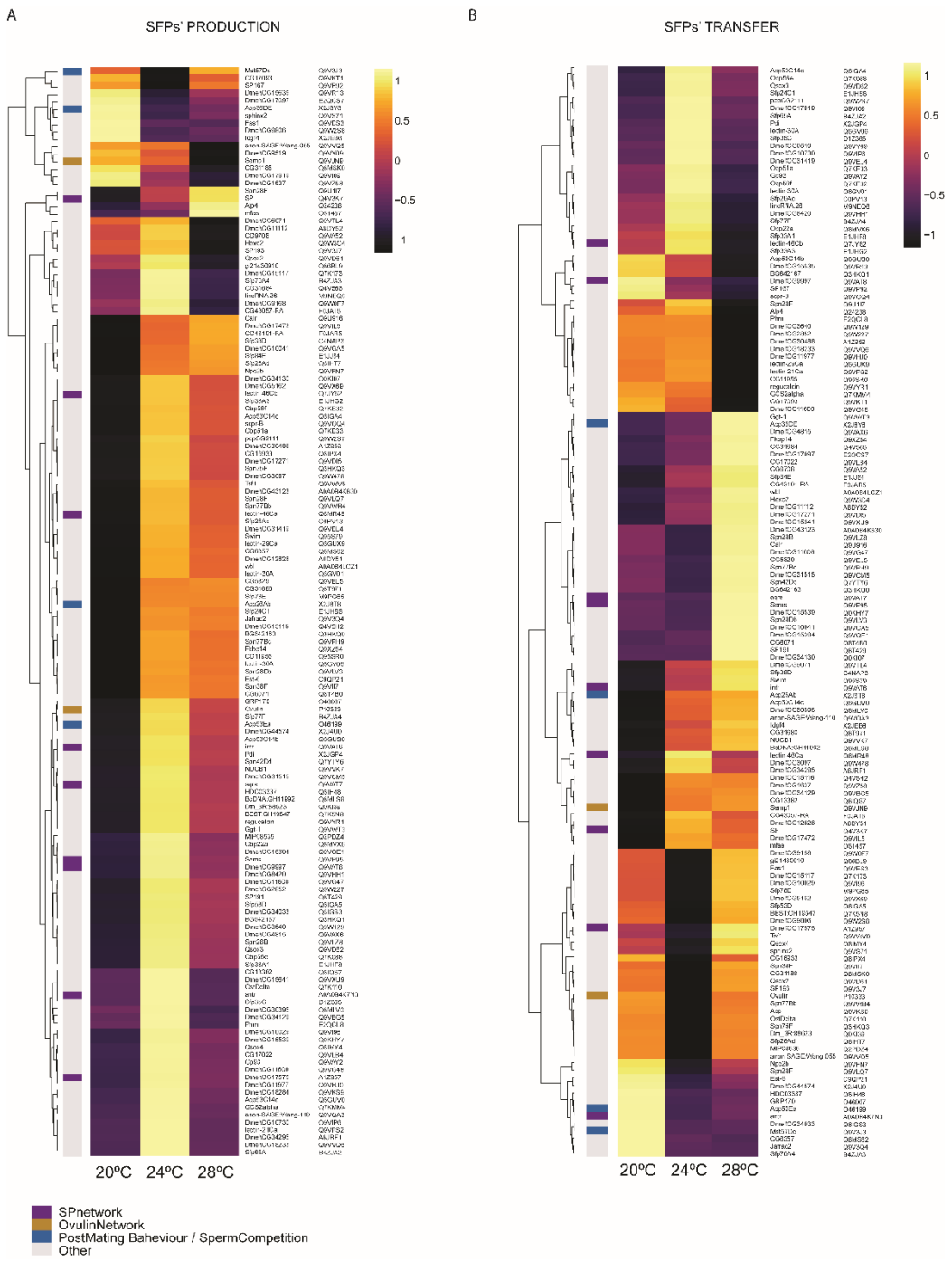


Figure 4 | Comparison of SFPs production and transfer under high-SCR vs. no-SCR across temperature treatments (short-term exposure / experiment 1). A) Comparison for production. B) Comparison for transfer. Each cell shows the mean ratio of protein absolute abundance under high-SCR relative to no-SCR across biological replicates for each temperature. Values closer to 1 indicate higher production/transfer under high-SCR conditions. Row annotations indicate functional information relating to protein functions as part of the sex peptide or ovulin networks, or other known roles in sperm competition.

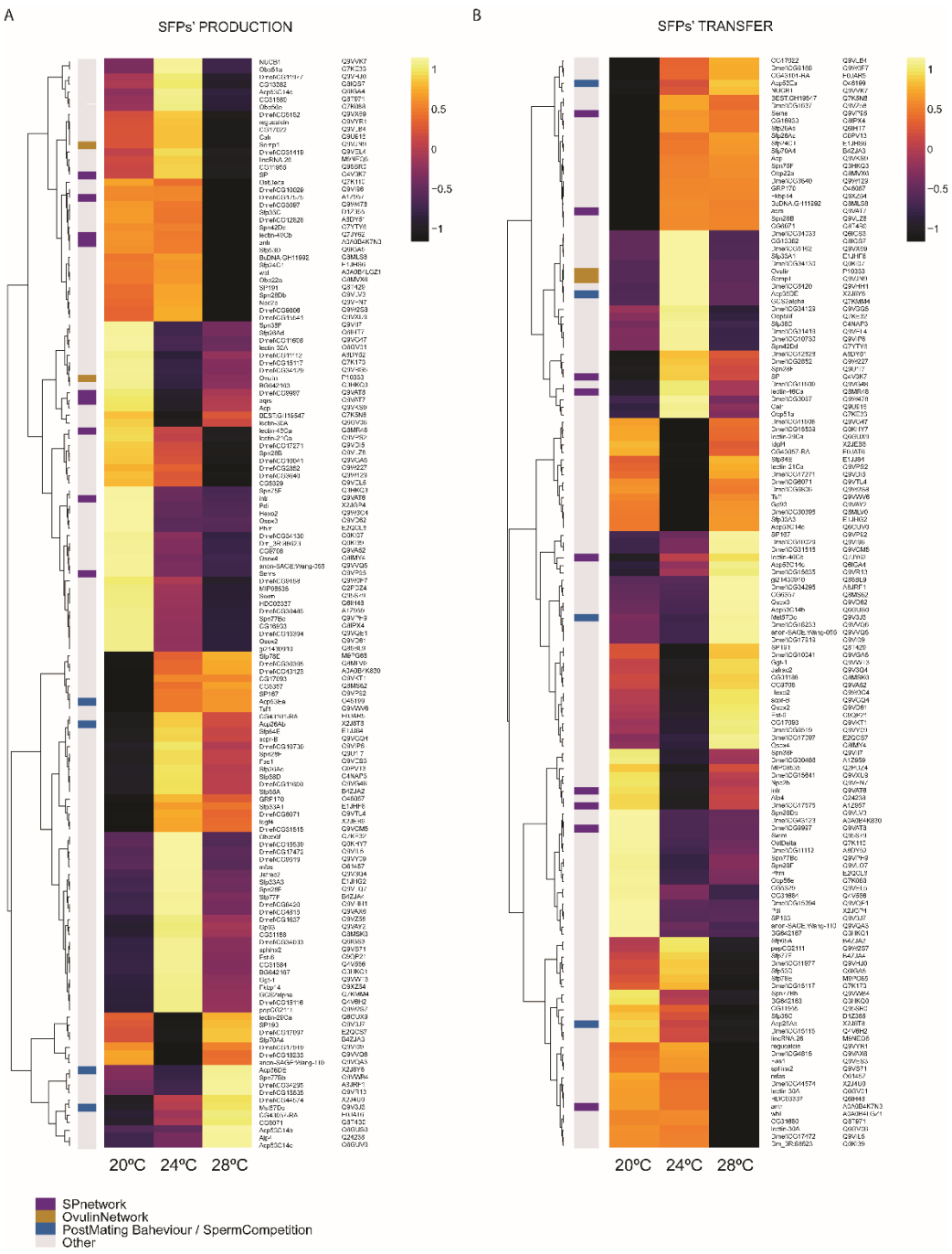
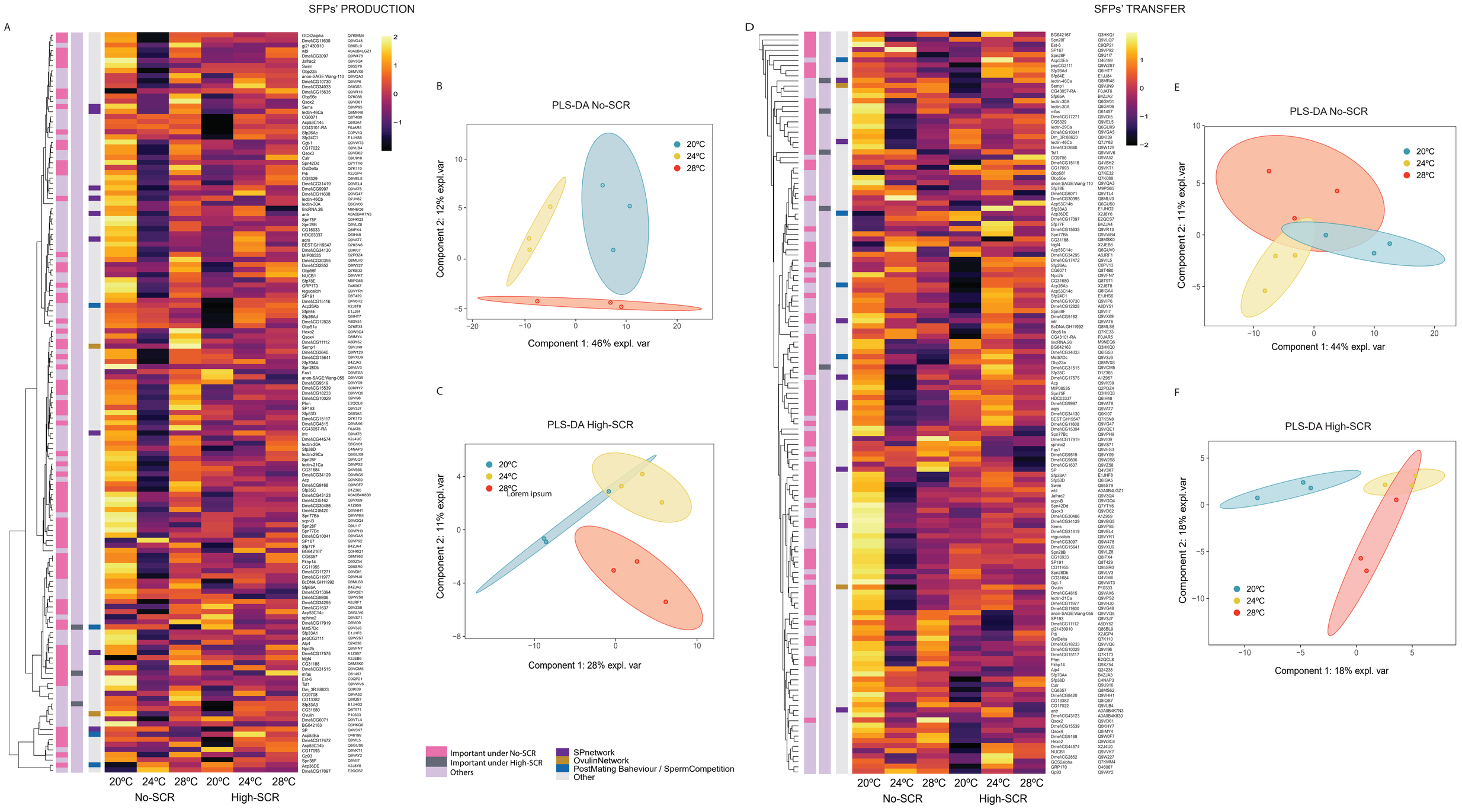
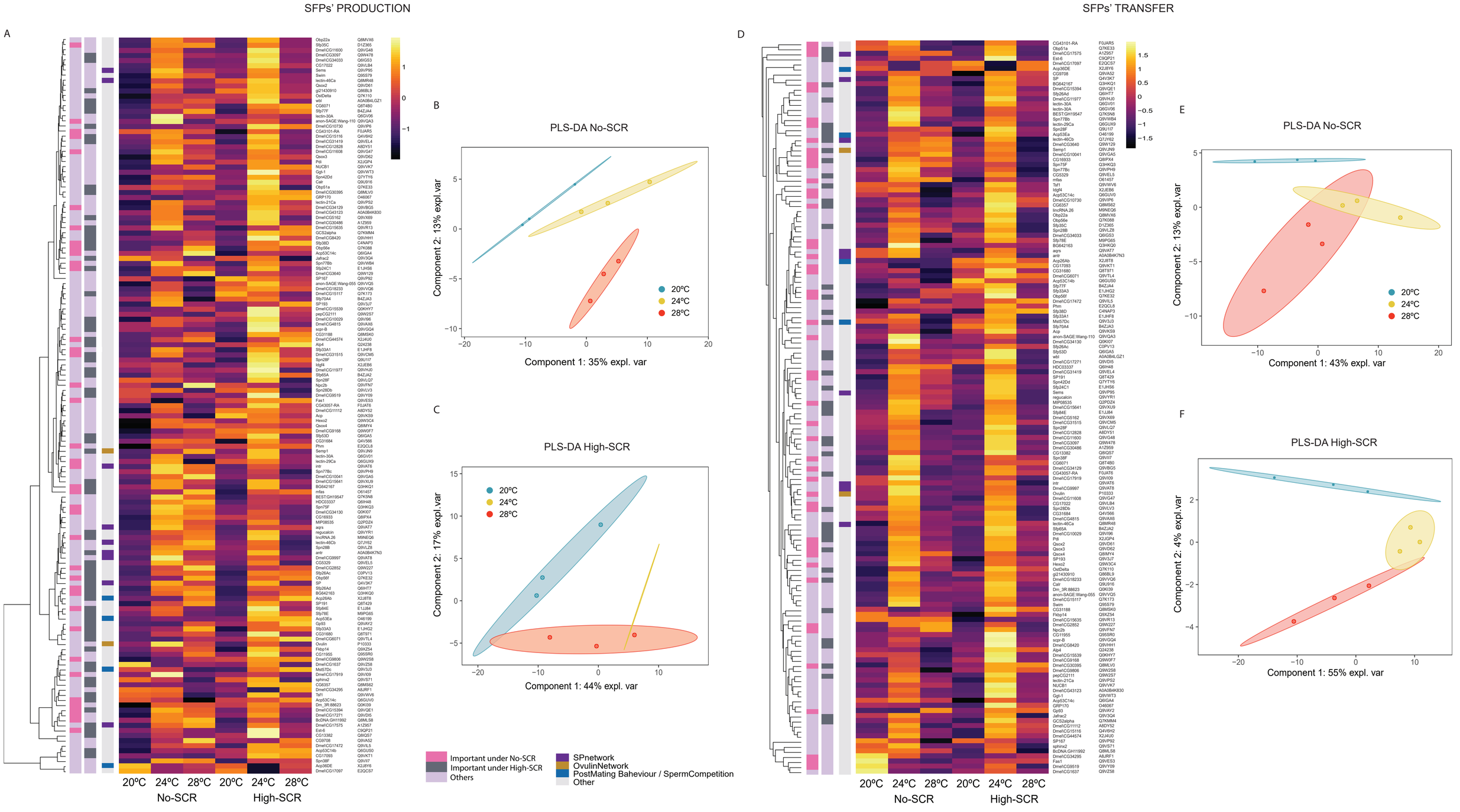
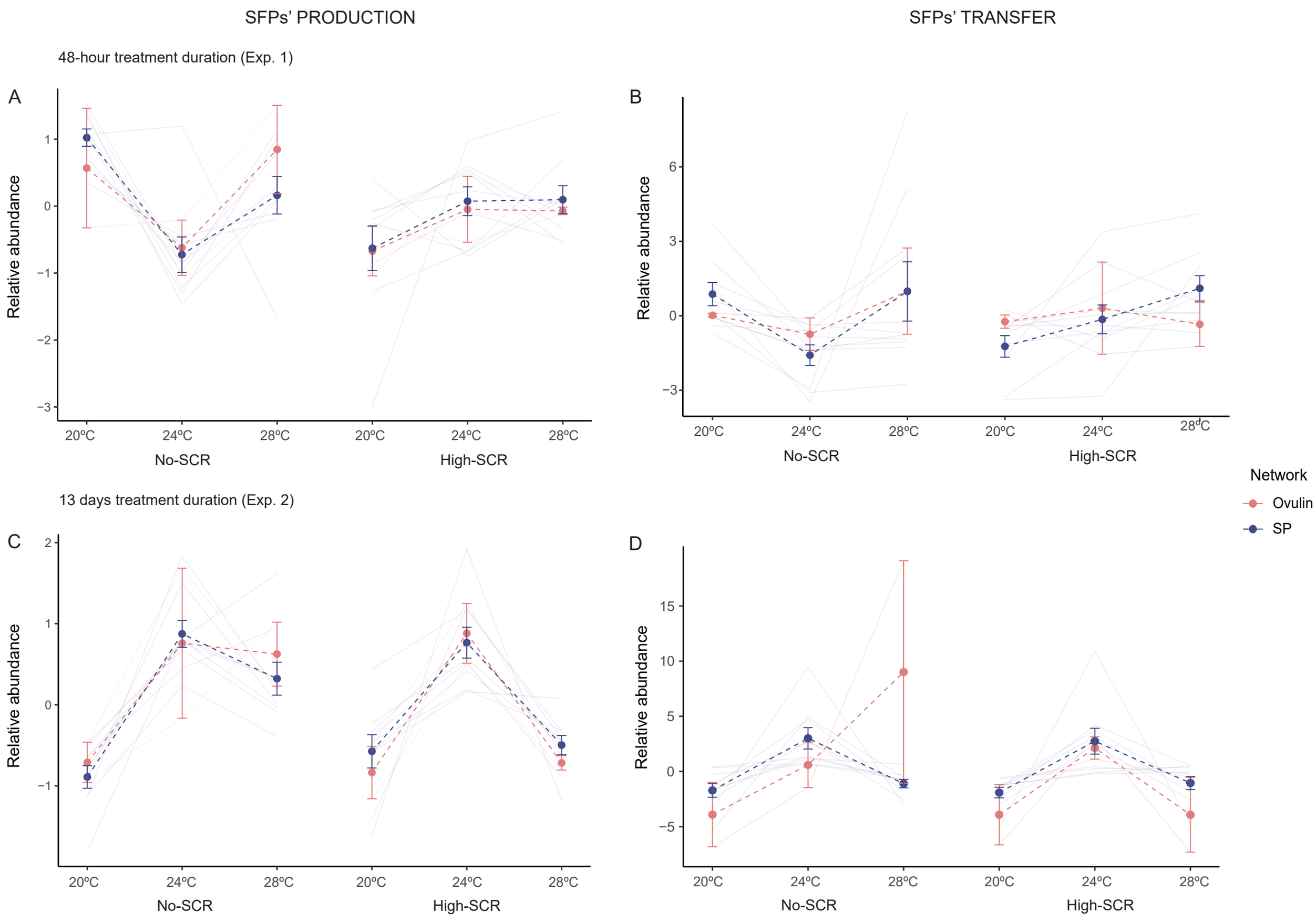
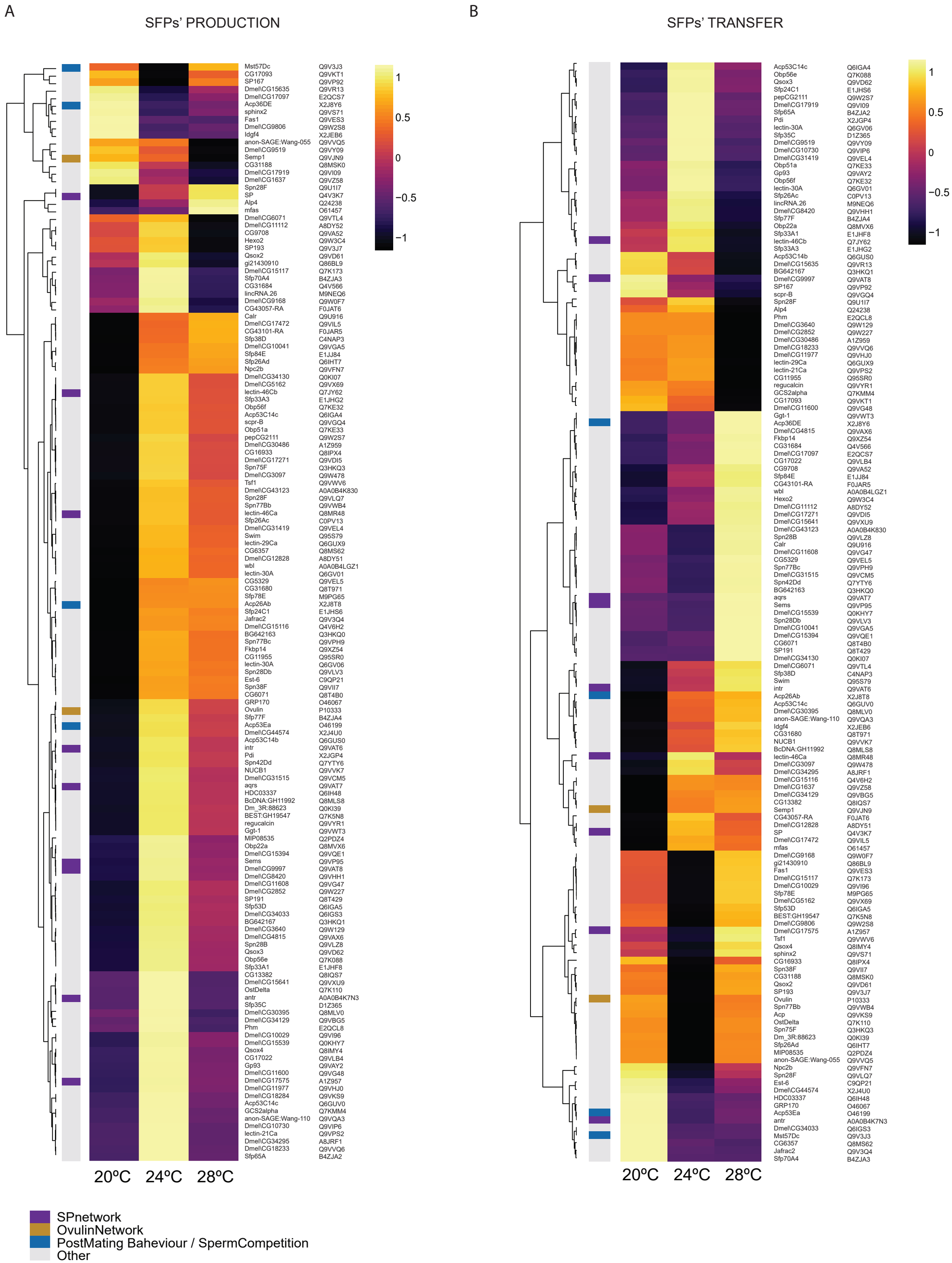


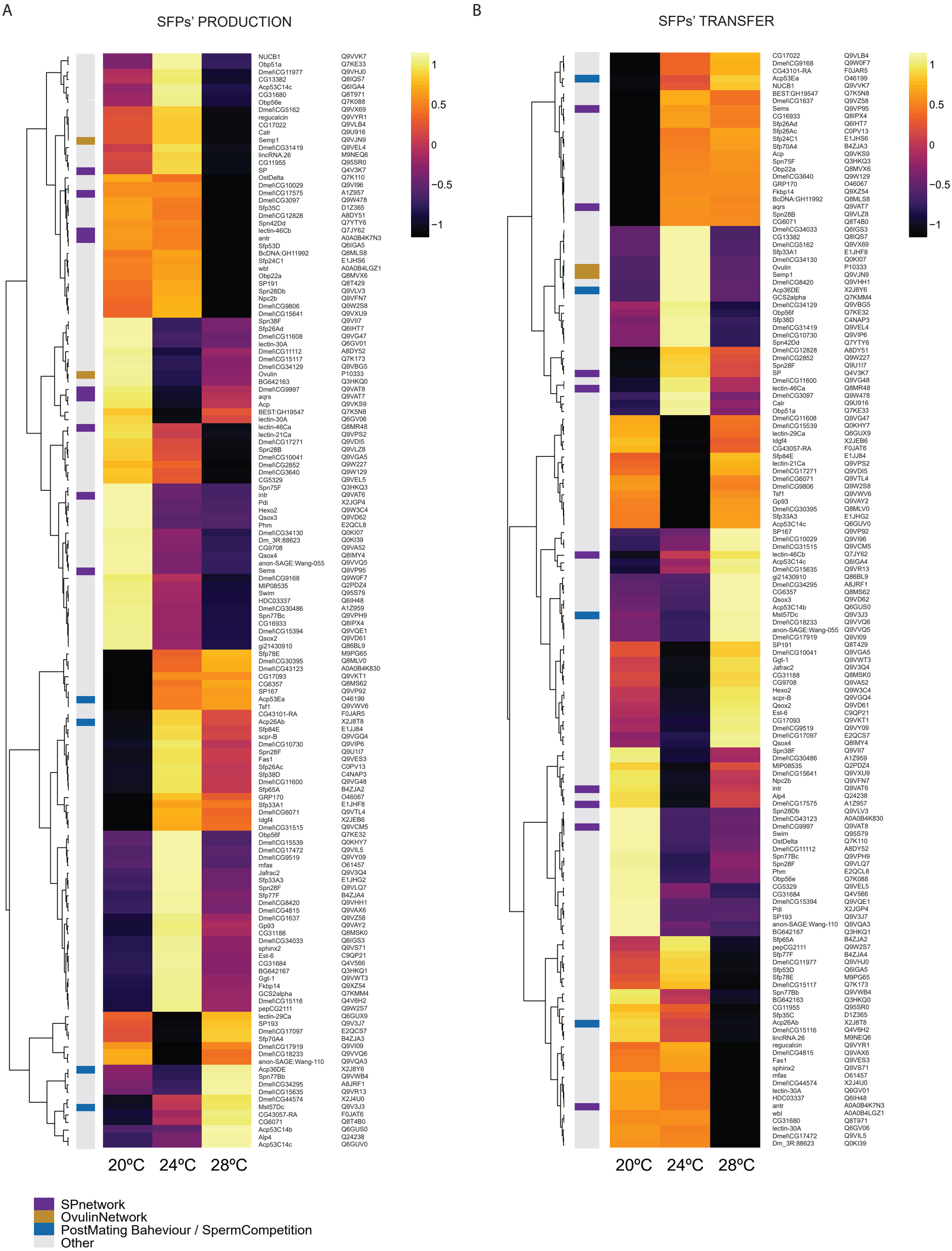
Figure 5 | Comparison of SFPs production and transfer under high-SCR vs. no-SCR across temperature treatments (long-term exposure / experiment 2). A) Comparison for production. B) Comparison for transfer. Each cell shows the mean ratio of protein absolute abundance under high-SCR relative to no-SCR across biological replicates for each temperature. Values closer to 1 indicate higher production/transfer under high-SCR conditions. Row annotations indicate functional information relating to protein functions as part of the sex peptide or ovulin networks, or other known roles in sperm competition.


SFPs' PRODUCTION
SFPs' TRANSFER
A
B
C
D
E
F
PLS-DA No-SCR
PLS-DA High-SCR
20ºC
24ºC
28ºC
Component 1: 46% expl. var
Component 2: 12% expl.var
Component 1: 28% expl. var
Component 2: 11% expl.var
Component 1: 44% expl. var
Component 2: 11% expl.var
Component 1: 18% expl. var
Component 2: 18% expl.var
Lorem ipsum
No-SCR
High-SCR
Important under No-SCR
Important under High-SCR
Others
SPnetwork
OvulinNetwork
PostMating Baheviour / SpermCompetition
Other


SFPs' PRODUCTION
SFPs' TRANSFER
A
B
C
D
E
F
PLS-DA No-SCR
PLS-DA High-SCR
Component 1: 35% expl. var
Component 2: 13% expl.var
Component 1: 44% expl. var
Component 2: 17% expl.var
Component 1: 43% expl. var
Component 2: 13% expl.var
Component 1: 55% expl. var
Component 2: 4% expl.var
20ºC
24ºC
28ºC
20ºC 24ºC 28ºC 20ºC 24ºC 28ºC
No-SCR
High-SCR
Important under No-SCR
Important under High-SCR
Others
SPnetwork
OvulinNetwork
PostMating Baheviour / SpermCompetition
Other


SFPs' PRODUCTION
SFPs' TRANSFER
48-hour treatment duration (Exp. 1)
A
B
Relative abundance
20ºC
24ºC
28ºC
No-SCR
High-SCR
13 days treatment duration (Exp. 2)
C
D
Network
Ovulin
SP


A
SFPs' PRODUCTION
B
SFPs' TRANSFER
1
0.5
0
−0.5
−1
20ºC
24ºC
28ºC
SPnetwork
OvulinNetwork
PostMating Baheviour / SpermCompetition
Other


A
SFPs' PRODUCTION
NUCB1 Q9VVK7
Obp51a Q7KE33
Dmel\CG11977 Q9VHJ0
CG13382 Q8IQS7
Acp53C14c Q6IGA4
CG31680 Q8T971
Obp56e Q7K088
Dmel\CG5162 Q9VX69
regucalcin Q9VYR1
CG17022 Q9VLB4
Calr Q9U916
Semp1 Q9VJN9
Dmel\CG31419 Q9VEL4
lincRNA.26 M9NEQ6
CG11955 Q95SR0
SP Q4V3K7
OstDelta Q7K110
Dmel\CG10029 Q9VI96
Dmel\CG17575 A1Z957
Dmel\CG3097 Q9W478
Sfp35C D1Z365
Dmel\CG12828 A8DY51
Spn42Dd Q7YTY6
lectin-46Cb Q7JY62
antr A0A0B4K7N3
Sfp53D Q6IGA5
BcDNA:GH11992 Q8MLS8
Sfp24C1 E1JHS6
wbl A0A0B4LGZ1
Obp22a Q8MVX6
SP191 Q8T429
Spn28Db Q9VLV3
Npc2b Q9VFN7
Dmel\CG9806 Q9W2S8
Dmel\CG15641 Q9VXU9
Spn38F Q9VII7
Sfp26Ad Q6IHT7
Dmel\CG11608 Q9VG47
lectin-30A Q6GV01
Dmel\CG11112 A8DY52
Dmel\CG15117 Q7K173
Dmel\CG34129 Q9VBG5
Ovulin P10333
BG642163 Q3HKQ0
Dmel\CG9997 Q9VAT8
aqrs Q9VAT7
Acp Q9VKS9
BEST:GH19547 Q7K5N8
lectin-30A Q6GV06
lectin-46Ca Q8MR48
lectin-21Ca Q9VPS2
Dmel\CG17271 Q9VDI5
Spn28B Q9VLZ8
Dmel\CG10041 Q9VGA5
Dmel\CG2852 Q9W227
Dmel\CG3640 Q9W129
CG5329 Q9VEL5
Spn75F Q3HKQ3
intr Q9VAT6
Pdi X2JGP4
Hexo2 Q9W3C4
Qsox3 Q9VD62
Phm E2QCL8
Dmel\CG34130 Q0KI07
Dm_3R:88623 Q0KI39
CG9708 Q9VA52
Qsox4 Q8IMY4
anon-SAGE:Wang-055 Q9VVQ5
Sems Q9VP95
Dmel\CG9168 Q9W0F7
MIP08535 Q2PDZ4
Swim Q95S79
HDC03337 Q6IH48
Dmel\CG30486 A1Z959
Spn77Bc Q9VPH9
CG16933 Q8IPX4
Dmel\CG15394 Q9VQE1
Qsox2 Q9VD61
gi21430910 Q86BL9
Sfp78E M9PG65
Dmel\CG30395 Q8MLV0
Dmel\CG43123 A0A0B4K830
CG17093 Q9VKT1
CG6357 Q8MS62
SP167 Q9VP92
Acp53Ea O46199
Tsf1 Q9VWV6
CG43101-RA F0JAR5
Acp26Ab X2J8T8
Sfp84E E1JJ84
scpr-B Q9VGQ4
Dmel\CG10730 Q9VIP6
Spn28F Q9U1I7
Fas1 Q9VES3
Sfp26Ac C0PV13
Sfp38D C4NAP3
Dmel\CG11600 Q9VG48
Sfp65A B4ZJA2
GRP170 O46067
Sfp33A1 E1JHF8
Dmel\CG6071 Q9VTL4
Idgf4 X2JEB6
Dmel\CG31515 Q9VCM5
Obp56f Q7KE32
Dmel\CG15539 Q0KHY7
Dmel\CG17472 Q9VIL5
Dmel\CG9519 Q9VY09
mfas O61457
Jafrac2 Q9V3Q4
Sfp33A3 E1JHG2
Spn28F Q9VLQ7
Sfp77F B4ZJA4
Dmel\CG8420 Q9VHH1
Dmel\CG4815 Q9VAX6
Dmel\CG1637 Q9VZ58
Gp93 Q9VAY2
CG31188 Q8MSK0
Dmel\CG34033 Q6IGS3
sphinx2 Q9VS71
Est-6 C9QP21
CG31684 Q4V566
BG642167 Q3HKQ1
Ggt-1 Q9VWT3
Fkbp14 Q9XZ54
GCS2alpha Q7KMM4
Dmel\CG15116 Q4V6H2
pepCG2111 Q9W2S7
lectin-29Ca Q6GUX9
SP193 Q9V3J7
Dmel\CG17097 E2QCS7
Sfp70A4 B4ZJA3
Dmel\CG17919 Q9VI09
Dmel\CG18233 Q9VVQ6
anon-SAGE:Wang-110 Q9VQA3
Acp36DE X2J8Y6
Spn77Bb Q9VWB4
Dmel\CG34295 A8JRF1
Dmel\CG15635 Q9VR13
Dmel\CG44574 X2J4U0
Mst57Dc Q9V3J3
CG43057-RA F0JAT6
CG6071 Q8T4B0
Acp53C14b Q6GUS0
Alp4 Q24238
Acp53C14c Q6GUV0
1
0.5
0
−0.5
−1
20ºC 24ºC 28ºC
B
SFPs' TRANSFER
CG17022 Q9VLB4
Dmel\CG9168 Q9W0F7
CG43101-RA F0JAR5
Acp53Ea O46199
NUCB1 Q9VVK7
BEST:GH19547 Q7K5N8
Dmel\CG1637 Q9VZ58
Sems Q9VP95
CG16933 Q8IPX4
Sfp26Ad Q6IHT7
Sfp26Ac C0PV13
Sfp24C1 E1JHS6
Sfp70A4 B4ZJA3
Acp Q9VKS9
Spn75F Q3HKQ3
Obp22a Q8MVX6
Dmel\CG3640 Q9W129
GRP170 O46067
Fkbp14 Q9XZ54
BcDNA:GH11992 Q8MLS8
aqrs Q9VAT7
Spn28B Q9VLZ8
CG6071 Q8T4B0
Dmel\CG34033 Q6IGS3
CG13382 Q8IQS7
Dmel\CG5162 Q9VX69
Sfp33A1 E1JHF8
Dmel\CG34130 Q0KI07
Ovulin P10333
Semp1 Q9VJN9
Dmel\CG8420 Q9VHH1
Acp36DE X2J8Y6
GCS2alpha Q7KMM4
Dmel\CG34129 Q9VBG5
Obp56f Q7KE32
Sfp38D C4NAP3
Dmel\CG31419 Q9VEL4
Dmel\CG10730 Q9VIP6
Spn42Dd Q7YTY6
Dmel\CG12828 A8DY51
Dmel\CG2852 Q9W227
Spn28F Q9U1I7
SP Q4V3K7
Dmel\CG11600 Q9VG48
lectin-46Ca Q8MR48
Dmel\CG3097 Q9W478
Calr Q9U916
Obp51a Q7KE33
Dmel\CG11608 Q9VG47
Dmel\CG15539 Q0KHY7
lectin-29Ca Q6GUX9
Idgf4 X2JEB6
CG43057-RA F0JAT6
Sfp84E E1JJ84
lectin-21Ca Q9VPS2
Dmel\CG17271 Q9VDI5
Dmel\CG6071 Q9VTL4
Dmel\CG9806 Q9W2S8
Tsf1 Q9VWV6
Gp93 Q9VAY2
Dmel\CG30395 Q8MLV0
Sfp33A3 E1JHG2
Acp53C14c Q6GUV0
SP167 Q9VP92
Dmel\CG10029 Q9VI96
Dmel\CG31515 Q9VCM5
lectin-46Cb Q7JY62
Acp53C14c Q6IGA4
Dmel\CG15635 Q9VR13
gi21430910 Q86BL9
Dmel\CG34295 A8JRF1
CG6357 Q8MS62
Qsox3 Q9VD62
Acp53C14b Q6GUS0
Mst57Dc Q9V3J3
Dmel\CG18233 Q9VVQ6
anon-SAGE:Wang-055 Q9VVQ5
Dmel\CG17919 Q9VI09
SP191 Q8T429
Dmel\CG10041 Q9VGA5
Ggt-1 Q9VWT3
Jafrac2 Q9V3Q4
CG31188 Q8MSK0
CG9708 Q9VA52
Hexo2 Q9W3C4
scpr-B Q9VGQ4
Qsox2 Q9VD61
Est-6 C9QP21
CG17093 Q9VKT1
Dmel\CG9519 Q9VY09
Dmel\CG17097 E2QCS7
Qsox4 Q8IMY4
Spn38F Q9VII7
Dmel\CG30486 A1Z959
MIP08535 Q2PDZ4
Dmel\CG15641 Q9VXU9
Npc2b Q9VFN7
intr Q9VAT6
Alp4 Q24238
Dmel\CG17575 A1Z957
Spn28Db Q9VLV3
Dmel\CG43123 A0A0B4K830
Dmel\CG9997 Q9VAT8
Swim Q95S79
OstDelta Q7K110
Dmel\CG11112 A8DY52
Spn77Bc Q9VPH9
Spn28F Q9VLQ7
Phm E2QCL8
Obp56e Q7K088
CG5329 Q9VEL5
CG31684 Q4V566
Dmel\CG15394 Q9VQE1
Pdi X2JGP4
SP193 Q9V3J7
anon-SAGE:Wang-110 Q9VQA3
BG642167 Q3HKQ1
Sfp65A B4ZJA2
pepCG2111 Q9W2S7
Sfp77F B4ZJA4
Dmel\CG11977 Q9VHJ0
Sfp53D Q6IGA5
Sfp78E M9PG65
Dmel\CG15117 Q7K173
Spn77Bb Q9VWB4
BG642163 Q3HKQ0
CG11955 Q95SR0
Sfp35C D1Z365
Acp26Ab X2J8T8
Dmel\CG15116 Q4V6H2
lincRNA.26 M9NEQ6
regucalcin Q9VYR1
Dmel\CG4815 Q9VAX6
Fas1 Q9VES3
sphinx2 Q9VS71
mfas O61457
Dmel\CG44574 X2J4U0
lectin-30A Q6GV01
HDC03337 Q6IH48
antr A0A0B4K7N3
wbl A0A0B4LGZ1
CG31680 Q8T971
lectin-30A Q6GV06
Dmel\CG17472 Q9VIL5
Dm_3R:88623 Q0KI39
1
0.5
0
−0.5
−1
20ºC 24ºC 28ºC
SPnetwork
OvulinNetwork
PostMating Baheviour / SpermCompetition
Other