# 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.

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

**Abstract**

Seminal fluid proteins (SFPs) are key mediators of post-mating interactions, shaping fertility, sperm use and female physiology with major consequences for both sexes. Because they often enhance male fitness while imposing costs on females, SFPs are central to sexual selection and sexual conflict. Their composition and allocation strategies are influenced by both environmental conditions and male state. However, how abiotic ecological factors regulate SFP expression, and how this interacts with sperm competition risk, remains poorly understood. Here, we use 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 to females. SFP production and transfer profiles were significantly affected by temperature, both consistently reduced after long-term (13d) exposure to low (20ºC) and high (28ºC) temperatures, compared to 24ºC. Proteins from the sex peptide and ovulin networks, which mediate female post-copulatory responses and are key to male fitness, followed this same pattern. Our results show that natural temperature fluctuations, well within the optimal reproductive range for flies in the wild, can have a strong effect on SFP production and transfer, and thus on post-copulatory sexual selection dynamics.

## 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, there are proteins that are crucial for SP and ovulin to function: *antr,*
61 *aqrs, intr, lectin-46Ca, lectin-46Cb, Sems, CG17575* and *CG9997*, contribute to the long-term
62 persistence of reduced female receptivity to remating, effective sperm release, and fecundity
63 stimulation (Findlay et al., 2014; Ravi Ram & Wolfner, 2007). *Semp1*, in turn, is necessary to
64 process the ovulin in mated females (Ravi Ram et al., 2006). Together, SP, ovulin and their
65 associated network proteins increase male reproductive success, and SP and its network proteins
66 are believed to be under sexual conflict, as they can substantially reduce female lifespan and long-
67 term reproductive output (Wigby & Chapman, 2005).

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

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

81 Temperature is a particularly salient ecological factor in this respect, due to its potentially
82 far-reaching effects on sexual reproduction and sexual selection/conflict processes (Dougherty et
83 al., 2024; García-Roa et al., 2020). In nature, temperature is subject to substantial spatiotemporal
84 variability at different scales (e.g. circadian, seasonal), potentially imposing strong selective
85 pressures on sperm and ejaculate traits related to sexual selection and reproductive performance,
86 especially in ectothermic species. For instance, high temperatures can impair gonad development,
87 reduce sperm production, viability and function, (Peña Jr. et al., 2019; Vasudeva et al., 2014; Wang
88 & Gunderson, 2022), and compromise protein stability and function (Berger et al., 2021). Indeed,
89 *D. melanogaster* SFPs are highly sensitive to heat stress (Canal Domenech & Fricke, 2022), and
90 recent evidence shows that, in this species, high temperatures affect SFP-induced female post-
91 mating responses and ensuing male harm levels (Londoño-Nieto et al., 2023). A small temperature
92 increase well within the optimal reproductive range of a natural population (i.e.from 24ºC to 28ºC,
93 where female lifetime reproductive success is equal across temperatures), is sufficient to reduce
94 both the impact of male ejaculates on female receptivity and overall male harm to females
95 (Londoño-Nieto et al., 2023). This finding is especially relevant, as it suggests that even mild,
96 naturally occurring temperature fluctuations may influence SFP production and transfer. Hence,
97 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).

Here, we used *D. melanogaster* flies to investigate how typical temperature variation, within the optimal reproductive range for a wild population, affects the production and transfer of SFPs, and how these effects change with male response to perceived sperm competition risk (hereafter SCR). We assessed these effects exposing adult males to temperature (i.e. 20ºC, 24ºC and 28ºC) and SCR treatments (i.e. no SCR vs. high SCR) across two different time scales (experiment 1: short-term exposure - 48 h; experiment 2: long-term exposure - 13 d).

We predicted that:

1) The seminal fluid proteome would be thermally plastic, such that SFP production and/or transfer would be reduced at high and low temperatures (20ºC and 28ºC). This is based on the hypothesis that male harm is mediated by SFPs, and harm was previously found to be reduced at 20ºC and 28ºC relative to 24ºC (Londoño-Nieto et al., 2023).
2) Long-term exposure to high temperatures (28ºC) would alter the seminal fluid proteome, especially the abundance of functionally important SFPs, (i.e. the sex peptide, ovulin and their network proteins), such that they would be produced and/or transferred in lower quantities. This is based on the finding that high temperatures reduce the impact of male ejaculates on female receptivity compared with lower temperatures (Londoño-Nieto et al., 2023).

## Materials and Methods

### Stock Maintenance

We carried out all experiments between November 2020 and March 2021, using individuals from our field-collected population, “Vegalibre”, established in October 2018 from three wineries in Requena (Spain), and re-sampled annually to maintain natural genetic variation (Londoño-Nieto et al., 2023). We keep Vegalibre in the laboratory with overlapping generations at an average temperature of 24°C with daily pre-programmed fluctuations (±4°C) mimicking natural daily temperature conditions during the reproductively active season, at ~60% humidity and on a 12:12 hr light:dark cycle. We used maize-malt medium (7 g of agar, 72 g of malt, 72 g of maize, 9 g of soya, 15 g of live yeast, 20 g of molasses, and 33 ml of Nipagin mix: 3 g of methyl 4-hydroxy-benzoate and 28 ml of ethanol 90% per 1000 ml of water) as a food source throughout maintenance and experiments. To collect experimental flies, we introduced yeasted grape juice agar plates into stock populations to induce female oviposition. We then collected eggs and placed them in bottles containing ~75 ml of medium to be incubated at 24 ± 4°C at a controlled density (Clancy & Kennington, 2001). We collected virgin flies within 6 hr of eclosion, under ice anesthesia, and then sexed and kept them in vials with food until their use (see below). All females used in our experiments were 4 days old, while males were 4 days old for experiment 1 and 16 days old for experiment 2, at the time when samples were collected.

<u>Proteomics experiments</u>

*Experimental overview and detailed methods*

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

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

147 In experiment 2, we examined longer-term temperature effects on the production and transfer of
148 newly synthesized SFPs that would be typical of seasonal effects. We took 2 days-old males and
149 first depleted their ejaculates by housing them individually with four virgin females for 24 hours,
150 since *D. melanogaster* males can become sperm and SFP depleted when mated sequentially over
151 a short period (Macartney et al., 2021; Sanghvi et al., 2025; Sirot et al., 2009). Then, we placed
152 the 3 days-old depleted males for 13 days in the same temperature-SCR treatments of the
153 experiment 1 (Fig. S2). Because males take at least 3 days to fully replenish their SFPs after
154 depletion (Sirot et al., 2009), this design ensured that males synthesized new SFPs under our
155 temperature treatments.

156 Immediately after treating males, we collected all samples at a common garden
157 temperature of 24ºC. To explore SFP production patterns, we measured the abundance of SFPs in
158 virgin (experiment 1) or unmated (for 13d, experiment 2) males across all temperature-SCR
159 treatment combinations. To explore SFP transfer patterns, we compared the abundance of SFPs
160 in virgin or unmated males with the abundance of SFPs in recently mated males across the same
161 treatment combinations (Hopkins, Sepil, Thézénas, et al., 2019; Sepil et al., 2019, 2020).

162 *Experimental matings*

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

175 We stored all frozen samples at -80°C until dissection, for which we thawed flash frozen
176 males and dissected their accessory glands, severing the ejaculatory duct and removing the
177 seminal vesicles and testes on ice in phosphate-buffered saline (PBS) under a binocular scope
178 (Hopkins, Sepil, Thézénas, et al., 2019). Each biological replicate (i.e. sample) consisted of a
179 pool of 20 reproductive glands in 25-µL PBS buffer. Samples were analysed with label-free
180 quantitative proteomics, using the sample preparation and quantification protocol (SWATH-MS;
181 Gillet et al., 2012) at the SCISIE proteomics service at the University of Valencia. Samples were
182 centrifuged and protein pellets resuspended in Laemmli buffer. Proteins were separated by SDS-
183 PAGE, full gel separation was performed for spectral library construction, while a short gel run
184 was used to clean and concentrate individual samples, followed by in-gel trypsin digestion as
185 described by Shevchenko et al. (1996). Peptides were analysed using a nanoESI qQTOF 6600+

186 TripleTOF mass spectrometer (AB SCIEX) coupled to a nanoflow LC system. Protein
187 identification and quantification were carried out using ProteinPilot and PeakView software with
188 a 1% false discovery rate threshold. Full methodological details are available in the
189 Supplementary Information. We analysed six samples per each temperature treatment-SCR
190 combination (three from virgin or unmated males and three from recently mated males). In total,
191 we had 72 samples (36 from experiment 1 and 36 from experiment 2).

192 Data analysis

193 We conducted all proteomics analysis on normalized abundances. We normalized the protein
194 areas calculated by the total sum of the areas of all the quantified proteins. We excluded proteins
195 with fewer than 2 unique peptides to restrict analysis to proteins with high-confidence
196 quantitation (Carr et al., 2004). We identified all proteins described as SFPs, based on a high-
197 confidence SFPs reference list from Wigby et al. (2020). We used an Elastic net penalized
198 logistic regression model and a partial least-squares discriminant analysis (PLS-DA) to analyse
199 our data sets, using *glmnet* (Friedman et al., 2010), *caret* (Kuhn, 2008) and *mixOmics* (Rohart et
200 al., 2017) packages in RStudio. Elastic net regression is a hybrid method that combines features
201 of Lasso and Ridge regularization (Zou & Hastie, 2005). It is particularly suited for situations
202 where the number of predictors far exceeds the number of observations, as it performs both
203 regularization and variable selection. The method applies a combined L1 (Lasso) and L2 (Ridge)
204 penalty that shrinks regression coefficients: weakly associated or irrelevant predictors are
205 penalized to exactly zero, effectively excluding them from the model, whereas variables with
206 stronger effects retain non-zero coefficients. Two parameters control the model: $\alpha$, which
207 balances the contribution of Lasso ($\alpha=1$) and Ridge ($\alpha=0$), and $\lambda$, which determines the overall
208 penalty strength. Both parameters were optimized using cross-validation (Zou & Hastie, 2005).

209 Under this framework, proteins with non-zero coefficients represent variables with the strongest
210 effects on proteome quantification.

211 To avoid overparameterization and maximize statistical power, we broke down analyses for both
212 experiments using the following scheme. To analyse how temperature affects SFP production
213 patterns (i.e. the abundance of SFPs in the accessory glands of virgin or unmated males), we
214 generated two datasets (one per SCR level), including all virgin (experiment 1) or unmated
215 (experiment 2) male samples across all temperature treatments. To analyse how temperature
216 influences SFP allocation, we first inferred the relative abundances of SFPs transferred by
217 dividing the abundance of each SFP in virgin or unmated males by its abundance in recently
218 mated males within the same temperature treatment, SCR level, and replicate. Based on these
219 ratios, we then generated two datasets (one per SCR level) including SFP transfer estimates
220 across all temperature treatments. To visualize the abundance of SFPs produced and transferred,
221 we took the average across the three biological replicates for each SFP within each temperature
222 treatment and SCR level. Heatmaps were generated from log2-transformed protein abundances,
223 using a Euclidean correlation distance metric and plotted with the aheatmap function from the
224 *NMF* package (Gaujoux & Seoighe, 2010) in RStudio (version R 4.2.2; R Core Team, 2022).

225 **Results**

226 We found a total of 1492 proteins, of which we retained 1328 for having 2 or more unique peptides.
227 Among these we focused on the 146 which were previously identified as SFPs.

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

229 Our results show that SFP production and transfer are highly plastic in response to temperature
230 variation across both treatment durations, but with important differences. In males treated for 48-

231 hour (short-term -experiment 1), elastic net regression identified 74 (under no-SCR) and 3 (under
232 high-SCR) SFPs as predictors contributing most strongly to temperature variation in protein
233 production (non-zero coefficients indicate proteins retained by the model; see Tables S2 and S3).
234 Similarly, the transfer of 79 (under no-SCR) and 6 (under high-SCR) SFPs was most strongly
235 associated with temperature variation (Tables S4 and S5). Heatmaps revealed that both production
236 and transfer of SFPs changed with temperature, with clearer effects under no-SCR. Partial least-
237 squares discriminant analysis (PLS-DA) supported these findings (Fig. 1), suggesting that SFP
238 production and transfer were more strongly affected by temperature in males exposed to no-SCR
239 than in those under high-SCR. Both the number of SFPs showing temperature-mediated changes
240 in abundance and the magnitude of these changes were markedly reduced under high-SCR (Fig.
241 1), suggesting that under high SCR male ejaculates are less thermally plastic after a short-term
242 exposure. In males treated for 13-days (long-term -experiment 2), elastic net regression selected
243 39 (under no-SCR) and 68 (under high-SCR) SFPs for production (Tables S6 and S7), and 49
244 (under no-SCR) and 46 (under high-SCR) SFPs for transfer (Tables S8 and S9) as predictors
245 contributing most strongly to differential response to temperature. Heatmap inspection showed
246 clear temperature-driven variation in SFP production and transfer, confirmed by the PLS-DA
247 analysis (Fig. 2). Overall, we found clear evidence that temperature effects after long-term
248 exposure were more uniform across SCR conditions, consistently reducing SPF production and
249 transfer at both low (20ºC) and hot (28ºC) temperatures (Fig. 2).

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

252 For proteins involved in female post-mating responses, we identified 8 members of the SP network
253 (*antr, aqrs, intr, lectin-46Ca, lectin-46Cb, Sems, CG17575* and *CG9997*) as well as SP itself, ovulin

254 and its precursor, *Semp1*. Our results show that at 20ºC and 28°C, the production and transfer of
255 these proteins, and specifically those involved in the SP network, frequently diverged from that
256 observed at 24ºC, with clearer effects after the long-term treatment duration (experiment 2).

257 In experiment 1 (short-term exposure), SFP production revealed a consistent pattern:
258 protein abundance at 24ºC was markedly reduced compared to 20ºC and 28ºC, but only under no-
259 SCR (Fig. 1A and 3A). Among the proteins selected as important, having the strongest contribution
260 to temperature variation responses, under no-SCR, *Sems, lectin-46Cb, CG17575,* all belonging to
261 the sex peptide (SP) network, followed this general pattern. *SP* itself, however, and *Semp1*, a
262 member of the ovulin network (Ravi Ram et al., 2006), were also selected as important proteins
263 but displayed different patterns, with SP decreasing and *Semp1* increasing at 28ºC relative to lower
264 temperatures (Figs. 1A and S3). In contrast, under high-SCR we did not identify a consistent
265 pattern. However *Mst57Dc,* a protein related to the regulation of female receptivity (Findlay et al.,
266 2008, 2009; Simmerl et al., 1995) was selected as important under both SCR treatments, but with
267 different patterns: it showed lower abundance at 28ºC under no-SCR, whereas under high-SCR its
268 lowest abundance was observed at 24ºC (Figs. 1A and S3), suggesting a complex interaction
269 between thermal and social cues. Consistent with differences in production, SFP transfer under no-
270 SCR was generally reduced at 24ºC compared to 20ºC and 28ºC, while we did not find a clear
271 temperature-dependent pattern under high-SCR (Fig. 1D and 3B). Moreover, *ovulin, SP* and
272 proteins associated with SP network (i.e. *lectin-46Cb, CG9997*) were among those selected as
273 important only under no-SCR, following the general pattern (Figs. 1D and S4). Under high-SCR,
274 *lectin-46Ca*, a SP-network protein, was the only SPF known to regulate female post-mating
275 physiology selected as important contributing to temperature variation responses, with higher
276 transfer at 28ºC compared to lower temperatures (Figs. 1D and S4).

277 In experiment 2 (long-term exposure), SFP production showed a clear trend of increased
278 production at 24ºC compared to 20ºC and 28ºC, regardless of SCR (Fig. 2A and 3C). SP and ovulin
279 network proteins (i.e., *Semp1*, *antr*, *SP*, *CG17575)* were only selected as important predictors in
280 males under high-SCR (Fig 2A), showing the general pattern of increased production at 24ºC (Figs
281 2A and S5). *Acp26Ab, Acp53Ea* and *Mst57Dc* were also selected as important proteins only in
282 males under high-SCR but with increased production at 28ºC compared with at lower temperatures
283 (Figs 2A and S5). Patterns of SFP transfer mirrored those observed for production. In both SCR
284 conditions, 24ºC yielded the highest transfer levels (Figs. 2D and 3D). Among proteins showing
285 temperature-dependent transfer, elastic net identified *Semp1*, *antr*, *CG17575, lectin-46Cb, Ovulin*
286 and *Mst57Dc* in males under no-SCR, and *Semp1*, *CG17575, lectin-46Cb, lectin-46Ca, Acp53Ea*
287 and *Mst57Dc* in males under high-SCR as the strongest contributors to temperature variation.
288 These proteins exhibited markedly higher transfer at 24ºC compared to 20ºC and 28º (Figs. 2D and
289 S4). Despite the general similarity across SCR levels, more proteins from the SP and ovulin
290 networks were identified by elastic net as strongly temperature-sensitive in terms of production
291 under high-SCR, while transfer patterns showed similar thermal response across SCR levels (Figs.
292 2A and 2D).

293 **Discussion**

294 In this study, we show that temperature variation, within the optimal reproductive range for a wild
295 *D. melanogaster* population, affects both the production and transfer of SFPs, with effects shaped
296 by SCR and treatment duration. First, our results show that SFP production and transfer are very
297 plastic in response to temperature. In particular, long-term (13d) exposure to both low (20ºC) and
298 high (28ºC) temperatures resulted in lower production and transfer of SFPs. Second, we show that
299 these effects carry over to proteins with known important functions in female post-mating

respondes, 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.

*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 both high and 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, with temperature effects on both the production and transfer being consistent across SCR levels. In the long-term exposure experiment, sexually mature males were exposed to different stable temperatures for 13-days following the depletion of accessory-glands via repeated matings (Macartney et al., 2021). While we cannot exclude the possibility that the number of females used to deplete male ejaculates (four) resulted in only a partial rather than full depletion (males can mate consecutively with up to 10 females; Sanghvi et al., 2025), males take at least 3 days to fully replenish their SFPs after depletion (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. Our results thus support the idea that seasonal changes in temperature may impose physiological constraints on the synthesis and/or transfer of SFPs, and potentially on associated trade-offs, that warrant further investigation. Furthermore, these effects fit well with the finding that male-induced harm to females is significantly reduced at 20ºC and 28ºC, as compared to 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 way that is not clearcut. Under no SCR, we found higher production and transfer at both low and high temperatures. Furthermore, these differences disappeared under high SCR. Our short-term experiment was designed to capture effects on a fine-grained ecological scale (i.e. intragenerational effects), given that sexually mature males were exposed to different stable temperatures for 48h and flies typically live considerably longer lifespans in the wild. Compared to our long-term treatment, we may expect temperature effects on the production of SFPs to be relatively limited in this treatment, as males experienced only a brief thermal exposure. One possibility is that the pattern observed under no-SCR in the short-term experiment may reflect compensatory plasticity, in which males transiently increase SFP production and transfer to counteract negative long-term thermal effects on SFPs at low and high temperatures (see above). Under high-SCR, previous studies indicate that males invest maximally in the production and transfer of SFP at 25ºC (Hopkins, Sepil, Thézénas, et al., 2019). This could explain why we find no differences across temperature treatments under high-SCR, if males invested maximally in SFP, making short-term thermal exposure to lower/higher temperatures insufficient to produce detectable changes. However, it is important to stress that this is, at this point, speculative and predicated on the assumption that short-term temperature effects on SFPs are adaptive, which may not be the case.

Broadly, our findings align with previous work showing that high temperatures disrupts several stages of protein synthesis, including transcription, translation, folding, and post-translational modification, which collectively affect protein functionality (Zheng et al., 2024). These disruptions are particularly detrimental to protein and sperm phenotype and function across

346 animal taxa (Dougherty et al., 2024; Reinhardt et al., 2015; Sales et al., 2018). For instance, heat
347 stress commonly reduces sperm production, motility, viability, and longevity (Wang & Gunderson,
348 2022). It also increases molecular entropy, which can interfere with correct protein folding and
349 decreases the proportion of functional proteins (Berger et al., 2021). Recent work has extended
350 these findings to seminal fluid components, suggesting that SFPs may also be thermally sensitive
351 (Canal Domenech & Fricke, 2022; Martinet et al., 2023). Our findings build on this work by
352 showing that non-stressful thermal variation within the optimal reproductive range can
353 significantly alter SFP production and transfer. The finding that even subtle and relatively fine-
354 grained temperature variation can have clear effects on SFP production and transfer opens up a
355 new avenue to understand how ecology affects sexual selection traits/dynamics, and may also shed
356 light on expected effects of climate change on populations. There is growing recognition that
357 fertility limits, rather than lethal temperature limits, may be more accurate predictors of extinction
358 vulnerability (Dougherty et al., 2024). For instance, some *Drosophila* species can become sterile
359 at temperatures well below their lethal limit (Parratt et al., 2021; Van Heerwaarden & Sgrò, 2021).
360 Our results suggest that even below these fertility thresholds, natural temperature fluctuations may
361 still influence sexual selection dynamics, potentially increasing vulnerability to climate change in
362 ways not yet fully appreciated (García-Roa et al., 2020).

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

365 Beyond the general effects of temperature on SFPs plasticity, we predicted that prolonged exposure
366 to 28ºC would particularly impact SFPs involved in the SP and ovulin networks based on the
367 evidence that the effect of male ejaculates on female remating behaviour is modulated by the
368 thermal environment males experienced prior to mating (Londoño-Nieto et al., 2023). Specifically,

369 ejaculates from males exposed during 13 days to 28°C induced a markedly weaker reduction in
370 female receptivity (i.e. shorter remating latency) compared to males kept at 20ºC and, especially,
371 at 24 °C (Londoño-Nieto et al., 2023). Our results support this prediction. After 13 days of
372 exposure, we observed consistently lower production and transfer of SP- and ovulin-associated
373 proteins at both 20ºC and 28ºC compared to 24ºC (Fig 2B).

374 Ejaculate composition is shaped by trade-offs between ejaculate components that mediate post-
375 mating sexual interactions and sperm competition (Perry et al., 2013). This, along with our results
376 rise an interesting question of whether temperature may affect males´ ability to strategically adjust
377 the production and transfer of SFPs in response to perceived SCR, where higher investment is
378 typically observed under high-SCR vs. no-SCR conditions (Hopkins, Sepil, Thézénas, et al., 2019;
379 Wigby et al., 2009). Although our design was not optimized to test this interaction directly, we
380 explored visual comparisons across temperature treatments to explore potential trends in
381 production and transfer of SFPs by males kept under high-SCR vs. no -SCR. We found a trend in
382 that high temperatures seem to disrupt the expected SCR-related patterns of SFP production and
383 transfer in both experiments, including proteins with key roles in female post-mating responses,
384 specifically those in the SP and ovulin networks (Figs. 4 and 5). At 28ºC, after short-term exposure,
385 the production of every member of both SP and ovulin networks (including ovulin but excluding
386 SP itself), was lower by males under high-SCR compared to males under no-SCR (Fig 4A).
387 Similarly, males under high-SCR transferred less *Semp1, lectin-46Cb, aqrs, ovulin, Sems, antr,*
388 *CG17575, CG9997,* and *SP* than those under no-SCR (Fig. 4B). After prolonged thermal exposure
389 at the same temperature (28ºC), males under high-SCR produced less *Semp1, lectin-46Cb, lectin-*
390 *46Ca, intr, Sems, antr, CG17575* and *SP* (Fig. 5A), and transferred less *intr, Sems, CG17575, lectin-*
391 *46Cb* and *Semp1* compared to males under no-SCR (Fig. 5B). Needless to say, this is just a trend

392 and thus must be taken as preliminary, but it suggests that temperature, and particularly high
393 temperatures, may limit males' ability to strategically adjust their ejaculate composition in
394 response to SCR. These results point to a potentially important interaction that warrants further
395 investigation.

396 *The ecological relevance of thermal effects on SFPs*

397 Taken together, our results underscore the broader implications of thermal sensitivity in ejaculate
398 composition. Due to the importance of SFPs that regulate key post-mating processes, their
399 plasticity (or lack thereof) under thermal variation could mediate individual fitness and shape
400 population responses to environmental change. Our results from experiment 1 suggest that short
401 term fine-scale temperature fluctuations (e.g. circadian) are unlikely to have major effects on
402 females and populations, especially under high SCR. However, our results form experiment
403 2capture the cumulative impact of thermal variation on ejaculate production and transfer over a
404 timeframe that spans both intragenerational and intergenerational seasonal effects. Such thermal
405 fluctuations are extremely common in the field, particularly in temperate climates, underscoring
406 the ecological relevance of our findings.

407 At the intragenerational scale, sustained temperature effects on continuous SFP production
408 and transfer are bound to alter male reproductive dynamics and overall reproductive success across
409 an individual's lifetime, potentially making plasticity itself a target of selection. Supporting this
410 idea, there is evidence showing that seminal fluid composition can vary among genotypes in
411 response to environmental conditions, with direct consequences for reproductive success (Patlar
412 & Ramm, 2020). Consistent with this, in the same wild *D. melanogaster* population used here, we
413 have detected strong GxE interactions in male reproductive success under thermal variation, with
414 individual genotypes exhibiting divergent, and sometimes opposing, quadratic reaction norms

415 (Londoño-Nieto et al., 2025). Interestingly, these GxE effects appear to be sexually dimorphic
416 (Panyam et al., unpublished results), indicating that males and females may experience, and
417 potentially adapt to, thermal variation in different ways. Moreover, experimental evolution under
418 sustained high-temperature regimes in this population revealed shifts in the abundance of several
419 SP- and ovulin-network proteins, suggesting that thermal environments can drive evolutionary
420 changes in SFPs composition (Londoño-Nieto et al., 2025).

421 At the intergenerational scale, prolonged periods of heat or cold may shift the targets of
422 post-copulatory sexual selection across seasons, potentially driving seasonal adaptive tracking
423 and/or balancing selection (Bergland et al., 2014; Hoffmann et al., 2005; Machado et al., 2021;
424 Rudman et al., 2022). For example, if higher temperatures consistently reduce the abundance or
425 effectiveness of key proteins like SP, this could weaken male ability to manipulate female post-
426 mating behaviour, potentially shifting the relative importance of pre- vs. post-copulatory sexual
427 selection towards the former. Moreover, as climate change increases the frequency and magnitude
428 of temperature fluctuations, such effects may be increasingly important for temperate populations
429 of ectothermic species.

430 **Conclusion**.

431 Our results show that natural temperature fluctuations can have a strong effect on SFP production
432 and transfer, and thus on post-copulatory sexual selection dynamics. We show that SFP production
433 and transfer are thermally plastic and modulated by sociosexual context and exposure duration.
434 Notably, long-term exposure to low and high temperatures consistently reduced the production and
435 transfer of SFPs, including key proteins involved in female post-mating responses. These findings
436 highlight how seasonal environmental variation can shape ejaculate traits under realistic ecological
437 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.

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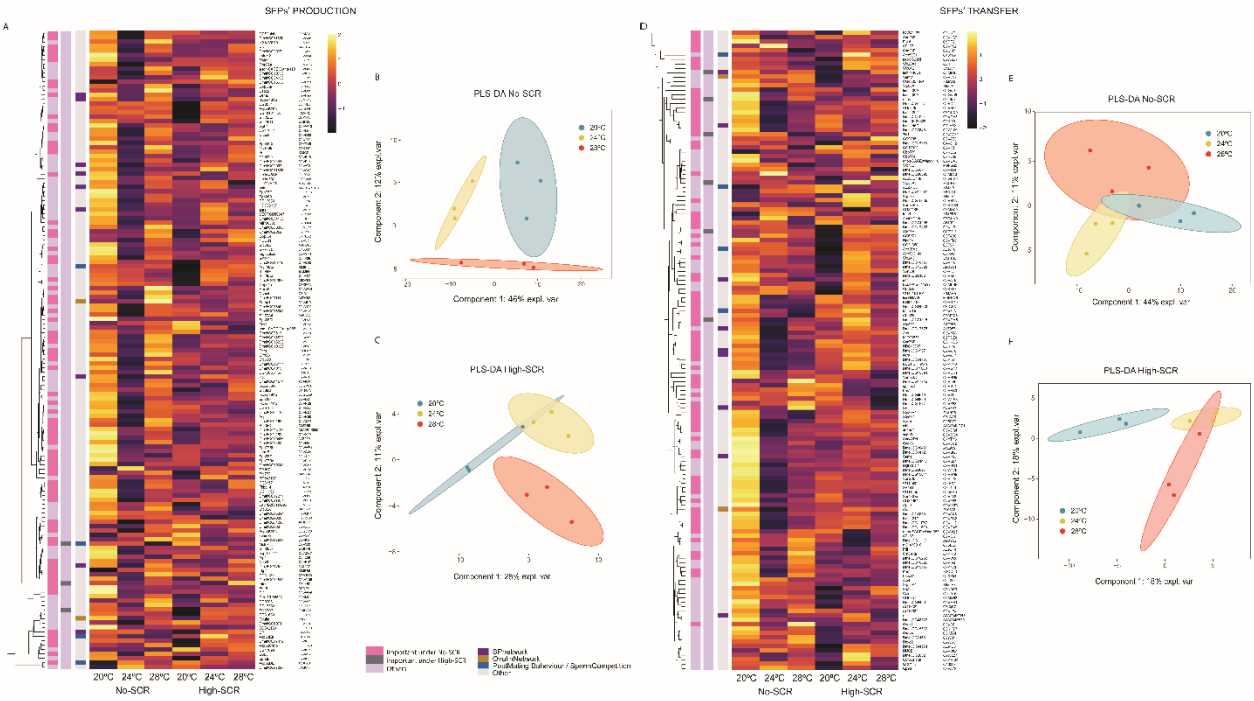



**Figure 1 | Effect of temperature on SFPs production and transfer (short-term exposure / experiment 1)**. A) Heatmap showing the abundance of 145 SFPs detected in accessory gland samples. Elastic net regression selected 74 (under no-SCR) and 3 (under High-SCR) out of 145 SFPs as predictors with a strong 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.

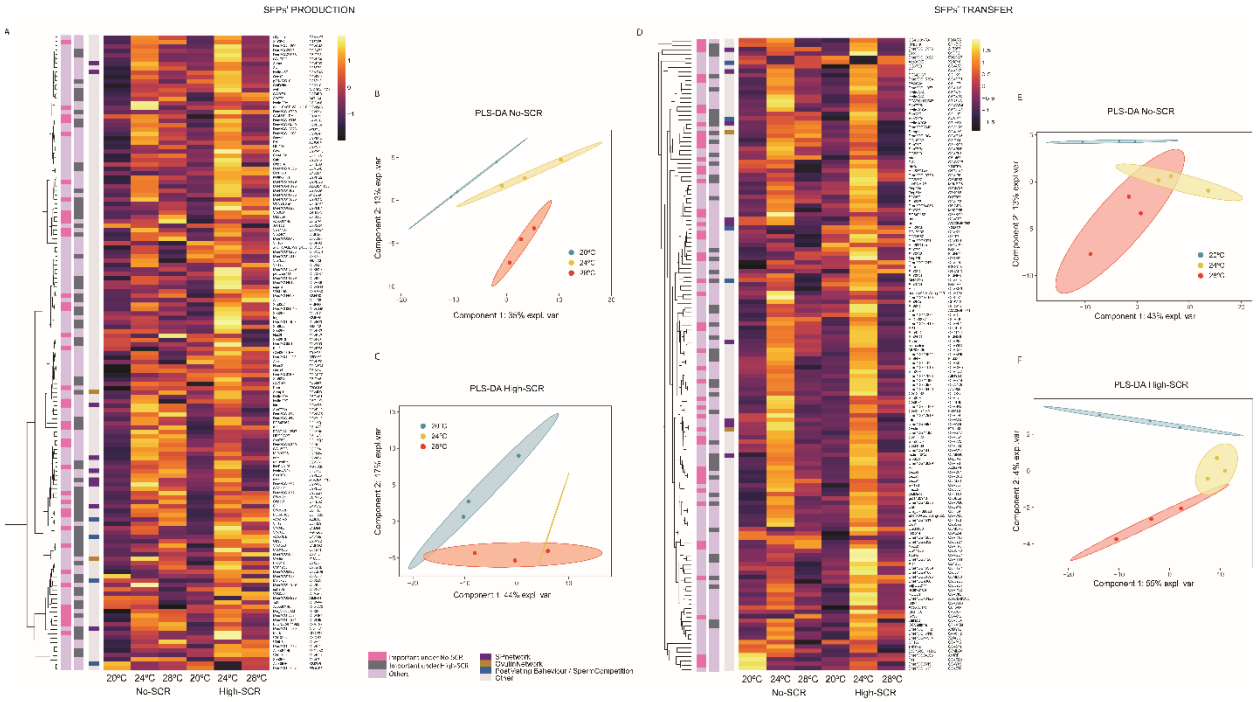



**Figure 2 | Effect of temperature on SFPs production and transfer (long-term exposure / experiment 2)**. A) Heatmap showing the abundance of the 145 SFPs detected in the accessory gland samples. Elastic net regression selected 39 (no SCR treatment) and 68 (High SCR treatment) out of 145 SFPs as predictors 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.

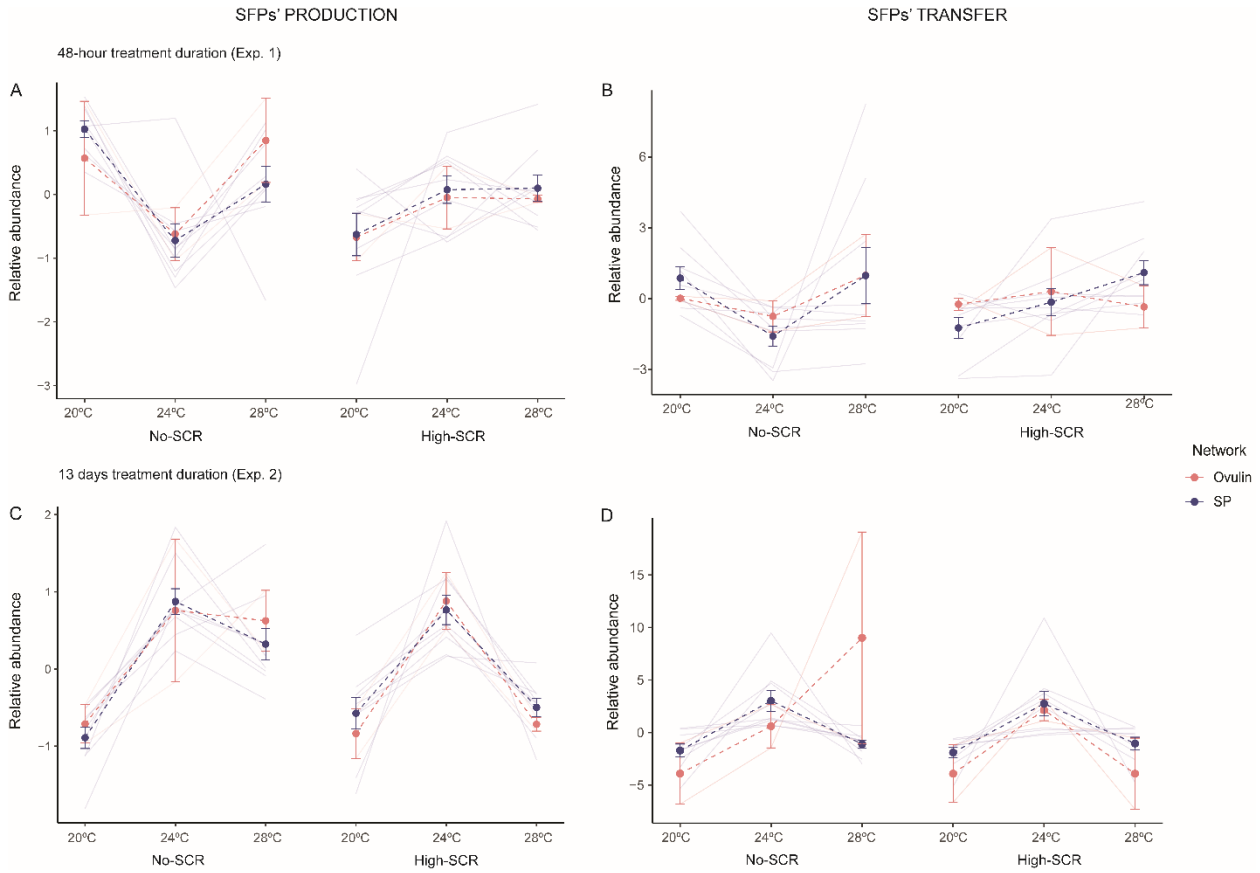
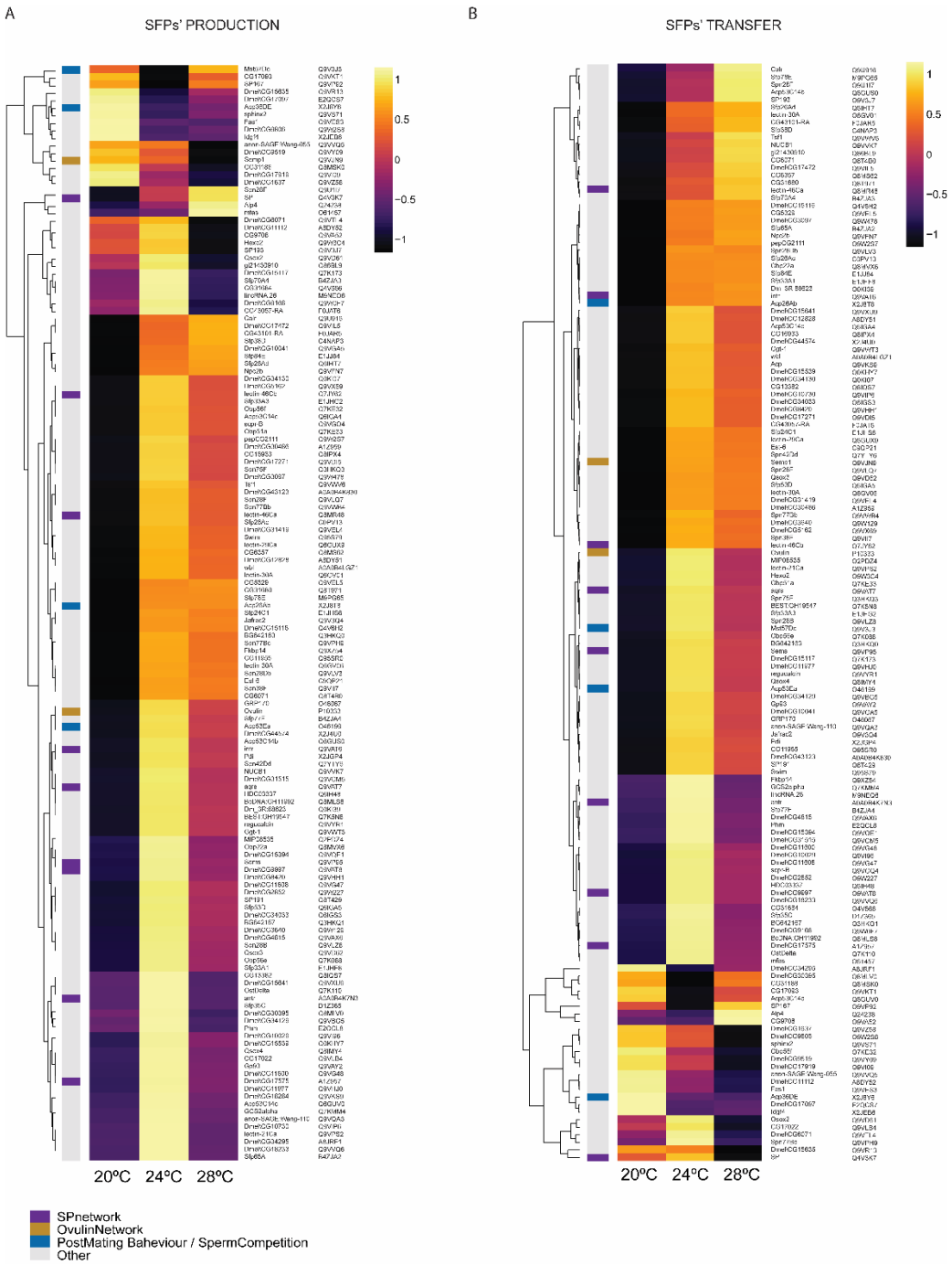



**Figure 3 | Effect of temperature on production and transfer of SFPs involved in Ovulin and SP networks**. A – B) Relative abundance production and transfer profiles of SPFs associated with the SP and ovulin networks across different temperatures and SCR levels for short-term exposure (experiment 1). 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.




751

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

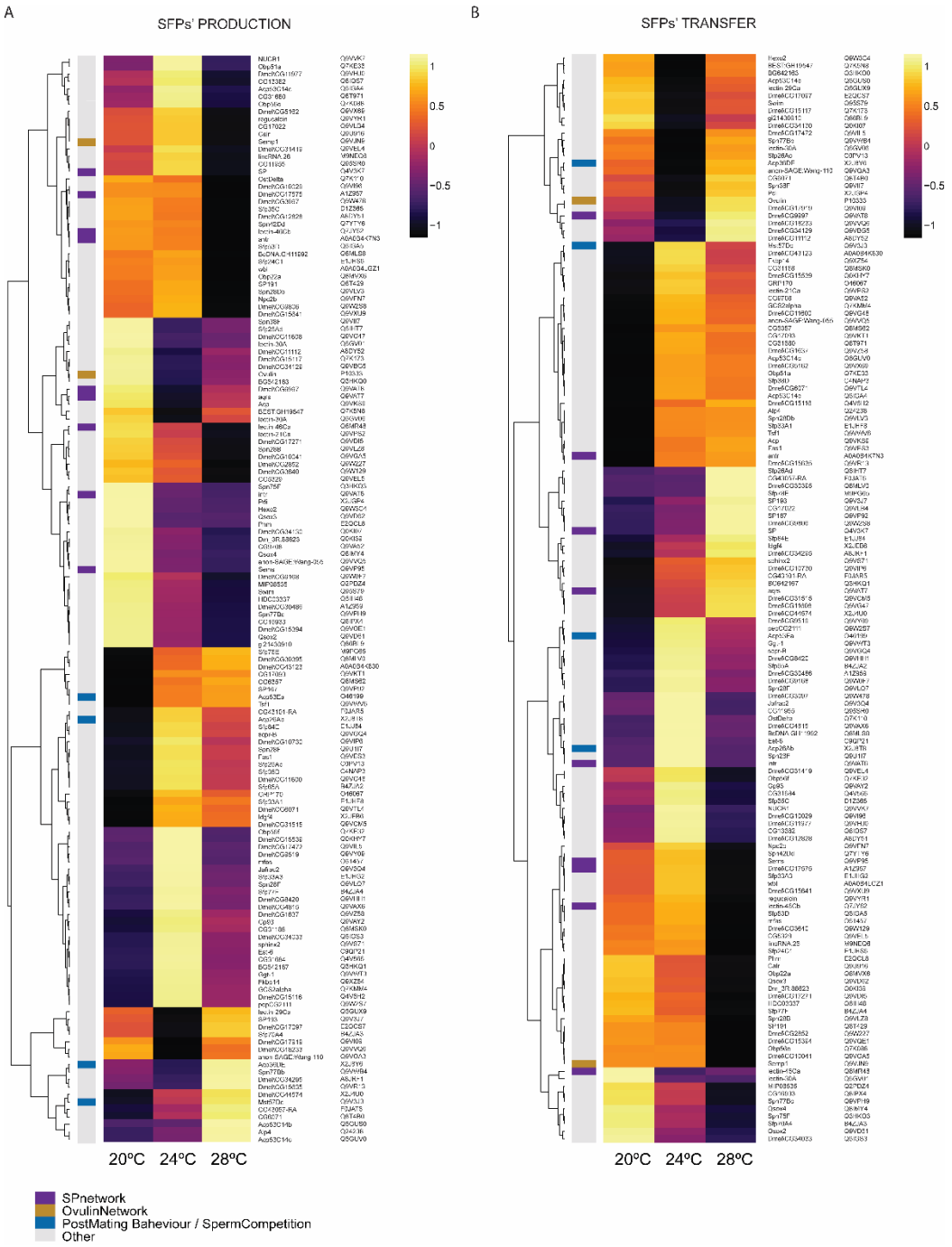
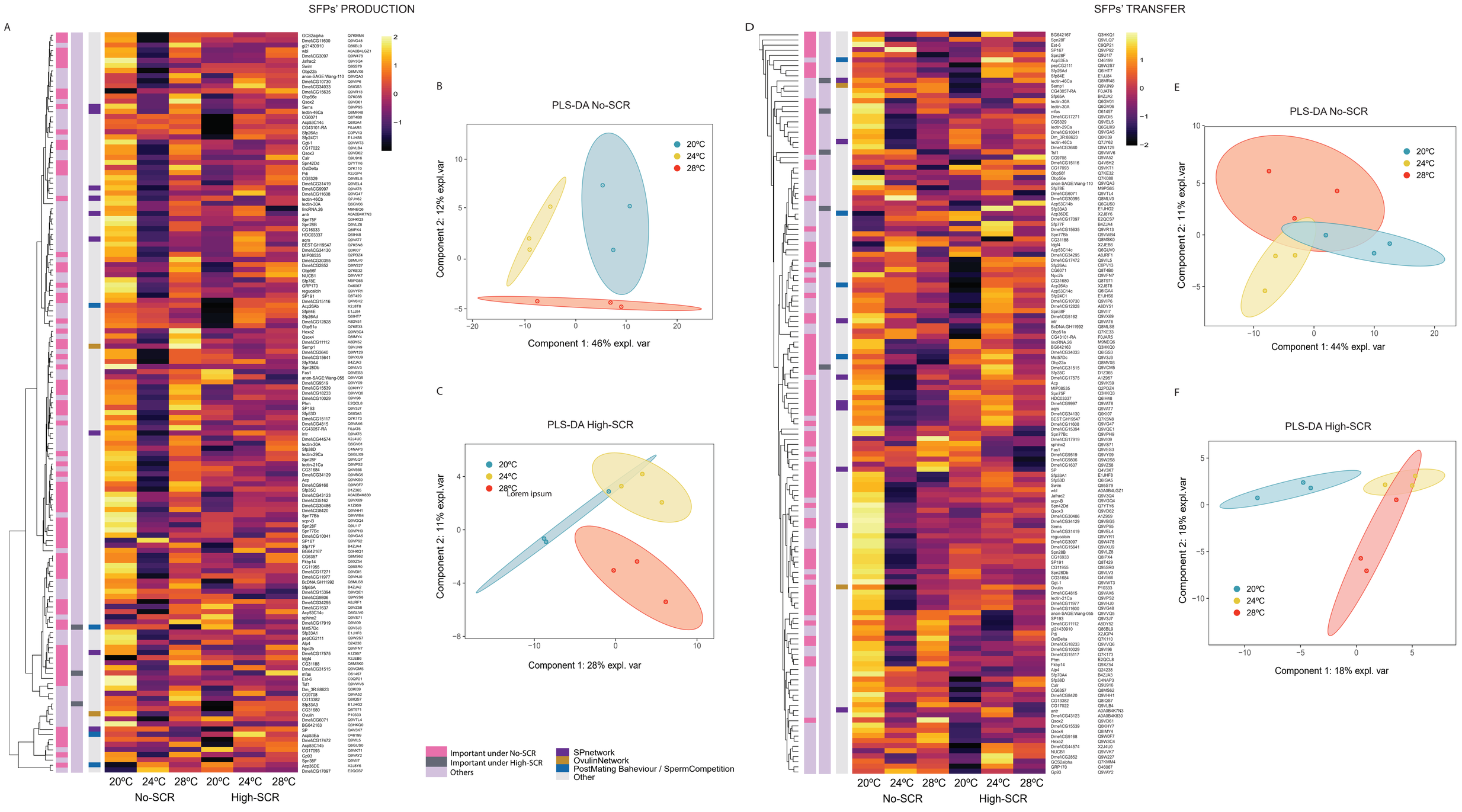
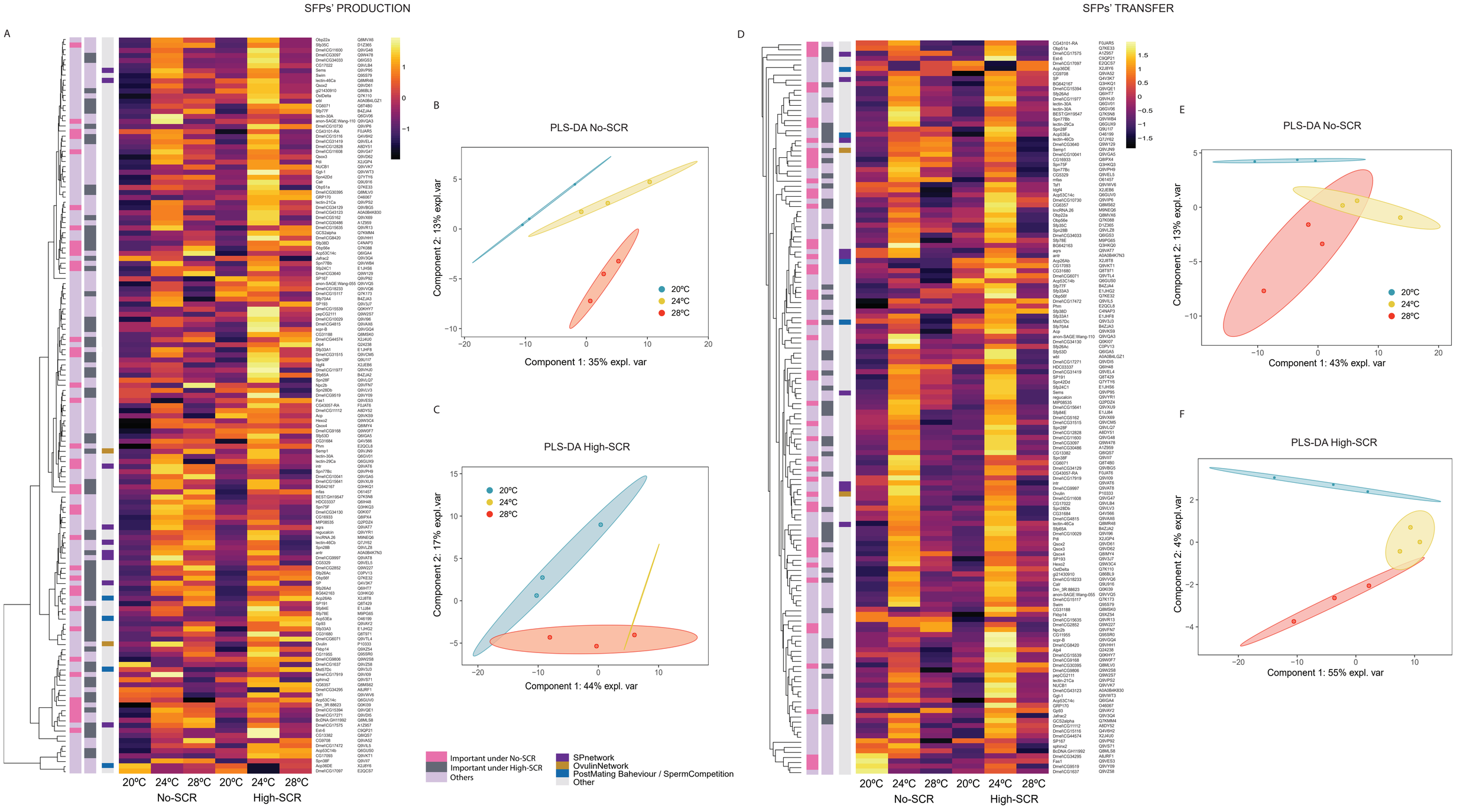
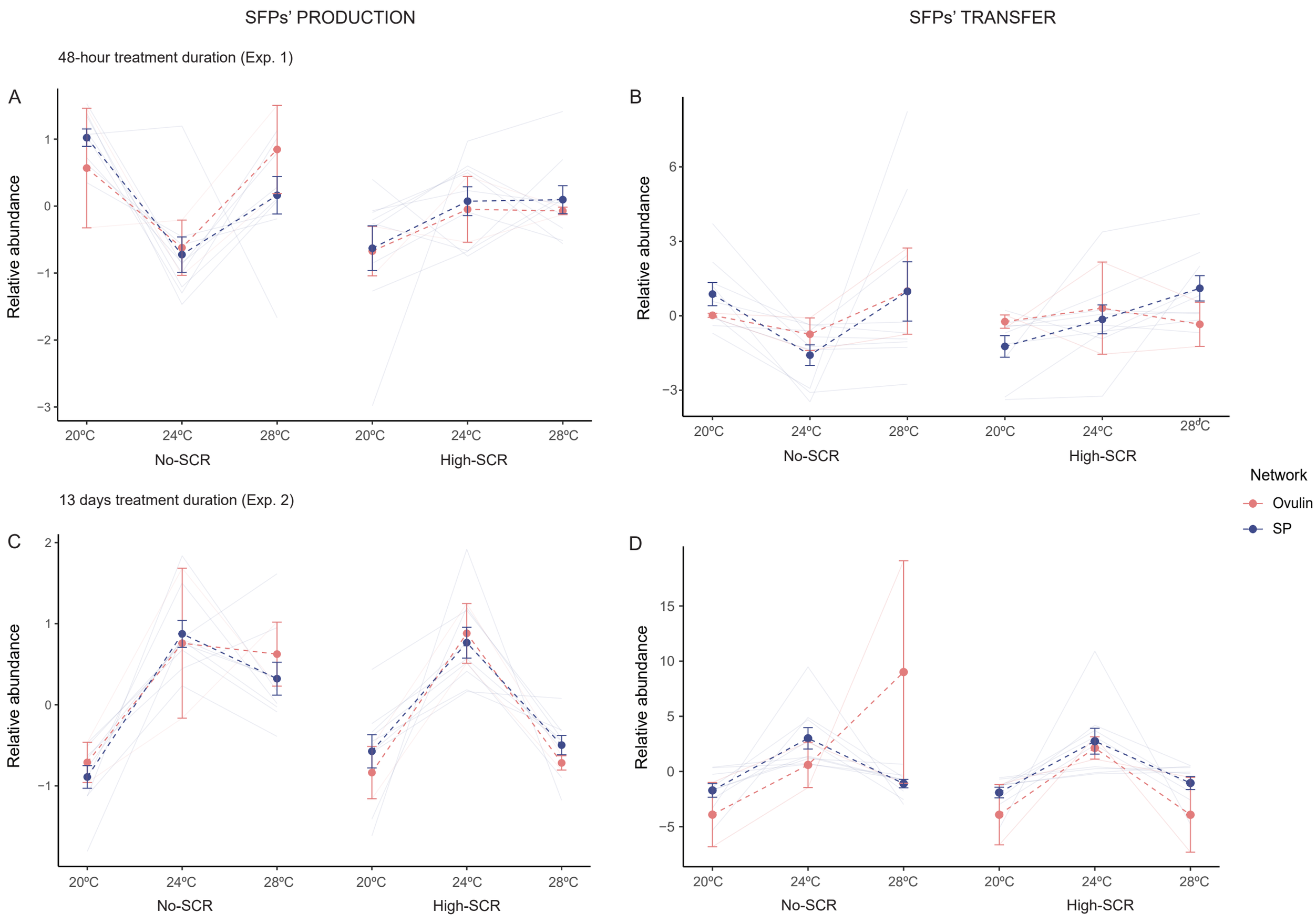
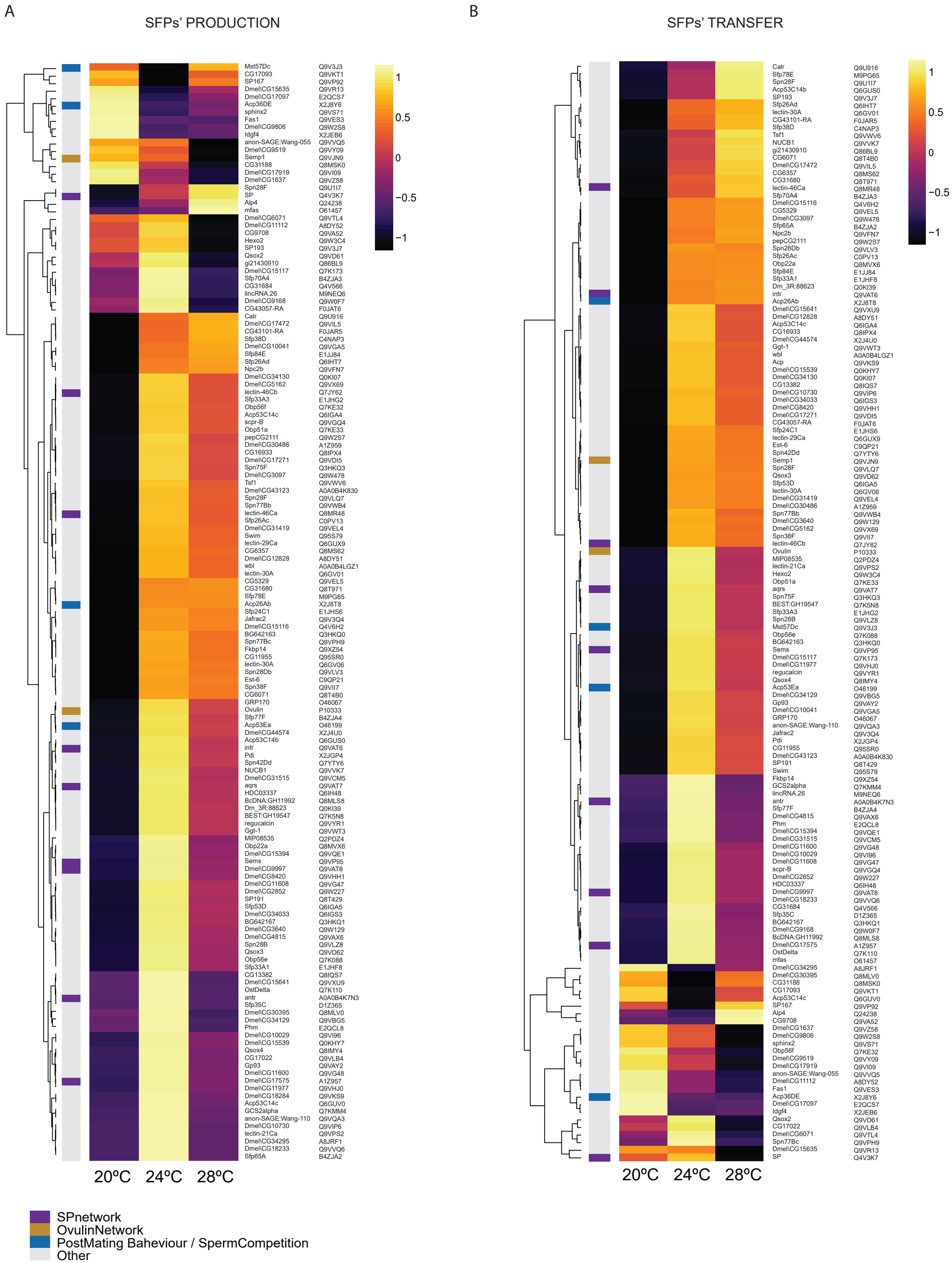



**Figure 5 | Comparison of SFPs production and transfer under high-SCR vs. no-SCR across temperature treatments (long-term exposure / experiment 2).** A) Comparison for production. B) Comparison for transfer. Each cell shows the mean ratio of protein abundance under high-SCR relative to no-SCR across 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
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SP167 Q9VP92
Dmel\CG15635 Q9VR13
Dmel\CG17097 E2QCS7
Acp36DE X2J8Y6
sphinx2 Q9VS71
Fas1 Q9VES3
Dmel\CG9806 Q9W2S8
Idgf4 X2JEB6
anon-SAGE:Wang-055 Q9VVQ5
Dmel\CG9519 Q9VY09
Semp1 Q9VJN9
CG31188 Q8MSK0
Dmel\CG17919 Q9VI09
Dmel\CG1637 Q9VZ58
Spn28F Q9U1I7
SP Q4V3K7
Alp4 Q24238
mfas O61457
Dmel\CG6071 Q9VTL4
Dmel\CG11112 A8DY52
CG9708 Q9VA52
Hexo2 Q9W3C4
SP193 Q9V3J7
Qsox2 Q9VD61
gi21430910 Q86BL9
Dmel\CG15117 Q7K173
Sfp70A4 B4ZJA3
CG31684 Q4V566
lincRNA.26 M9NEQ6
Dmel\CG9168 Q9W0F7
CG43057-RA F0JAT6
Calr Q9U916
Dmel\CG17472 Q9VIL5
CG43101-RA F0JAR5
Sfp38D C4NAP3
Dmel\CG10041 Q9VGA5
Sfp84E E1JJ84
Sfp26Ad Q6IHT7
Npc2b Q9VFN7
Dmel\CG34130 Q0KI07
Dmel\CG5162 Q9VX69
lectin-46Cb Q7JY62
Sfp33A3 E1JHG2
Obp56f Q7KE32
Acp53C14c Q6IGA4
scpr-B Q9VGQ4
Obp51a Q7KE33
pepCG2111 Q9W2S7
Dmel\CG30486 A1Z959
CG16933 Q8IPX4
Dmel\CG17271 Q9VDI5
Spn75F Q3HKQ3
Dmel\CG3097 Q9W478
Tsf1 Q9VWV6
Dmel\CG43123 A0A0B4K830
Spn28F Q9VLQ7
Spn77Bb Q9VWB4
lectin-46Ca Q8MR48
Sfp26Ac C0PV13
Dmel\CG31419 Q9VEL4
Swim Q95S79
lectin-29Ca Q6GUX9
CG6357 Q8MS62
Dmel\CG12828 A8DY51
wbl A0A0B4LGZ1
lectin-30A Q6GV01
CG5329 Q9VEL5
CG31680 Q8T971
Sfp78E M9PG65
Acp26Ab X2J8T8
Sfp24C1 E1JHS6
Jafrac2 Q9V3Q4
Dmel\CG15116 Q4V6H2
BG642163 Q3HKQ0
Spn77Bc Q9VPH9
Fkbp14 Q9XZ54
CG11955 Q95SR0
lectin-30A Q6GV06
Spn28Db Q9VLV3
Est-6 C9QP21
Spn38F Q9VII7
CG6071 Q8T4B0
GRP170 O46067
Ovulin P10333
Sfp77F B4ZJA4
Acp53Ea O46199
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Acp53C14b Q6GUS0
intr Q9VAT6
Pdi X2JGP4
Spn42Dd Q7YTY6
NUCB1 Q9VVK7
Dmel\CG31515 Q9VCM5
aqrs Q9VAT7
HDC03337 Q6IH48
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Dm_3R:88623 Q0KI39
BEST:GH19547 Q7K5N8
regucalcin Q9VYR1
Ggt-1 Q9VWT3
MIP08535 Q2PDZ4
Obp22a Q8MVX6
Dmel\CG15394 Q9VQE1
Sems Q9VP95
Dmel\CG9997 Q9VAT8
Dmel\CG8420 Q9VHH1
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Dmel\CG2852 Q9W227
SP191 Q8T429
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CG13382 Q8IQS7
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OstDelta Q7K110
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Sfp35C D1Z365
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Dmel\CG34129 Q9VBG5
Phm E2QCL8
Dmel\CG10029 Q9VI96
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Qsox4 Q8IMY4
CG17022 Q9VLB4
Gp93 Q9VAY2
Dmel\CG11600 Q9VG48
Dmel\CG17575 A1Z957
Dmel\CG11977 Q9VHJ0
Dmel\CG18284 Q9VKS9
Acp53C14c Q6GUV0
GCS2alpha Q7KMM4
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lectin-21Ca Q9VPS2
Dmel\CG34295 A8JRF1
Dmel\CG18233 Q9VVQ6
Sfp65A B4ZJA2
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0.5
0
−0.5
−1
20ºC 24ºC 28ºC
B
SFPs' TRANSFER
Calr Q9U916
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Acp53C14b Q6GUS0
SP193 Q9V3J7
Sfp26Ad Q6IHT7
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CG43101-RA F0JAR5
Sfp38D C4NAP3
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CG6071 Q8T4B0
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CG6357 Q8MS62
CG31680 Q8T971
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CG5329 Q9VEL5
Dmel\CG3097 Q9W478
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Npc2b Q9VFN7
pepCG2111 Q9W2S7
Spn28Db Q9VLV3
Sfp26Ac C0PV13
Obp22a Q8MVX6
Sfp84E E1JJ84
Sfp33A1 E1JHF8
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CG17022 Q9VLB4
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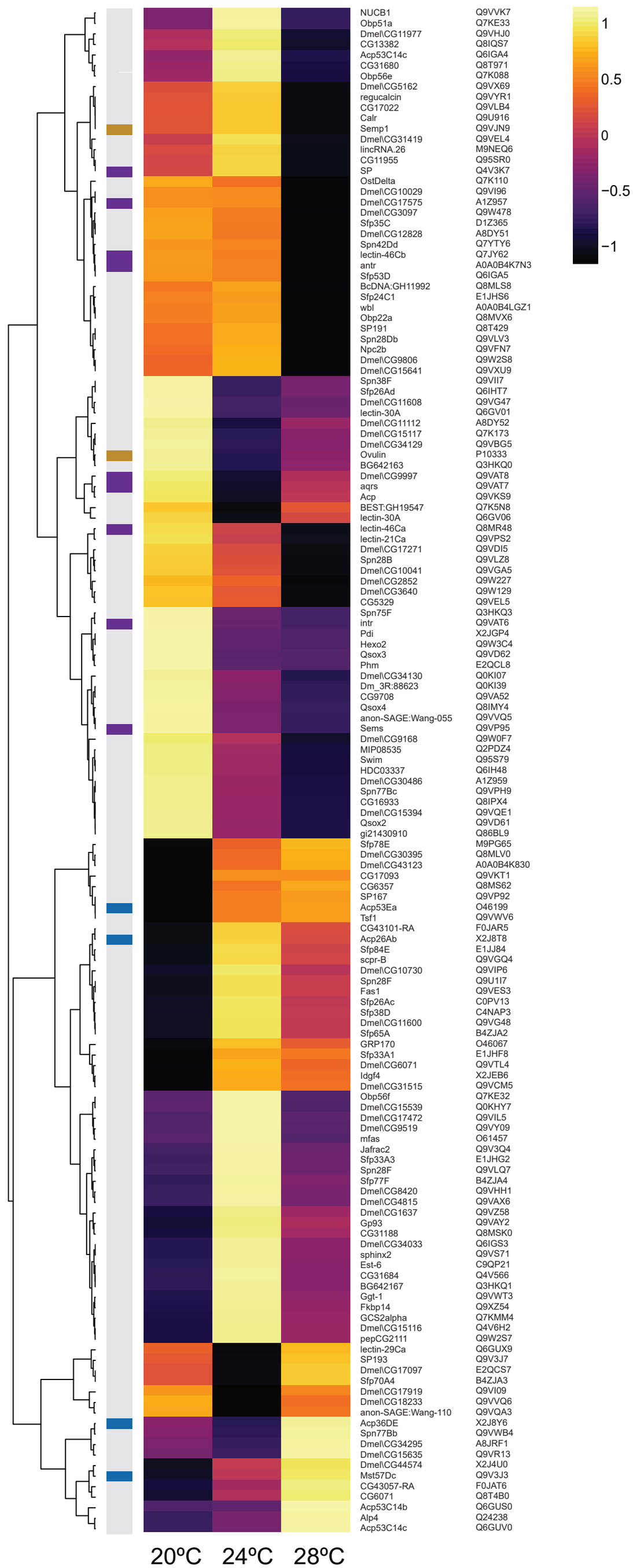
OvulinNetwork
PostMating Baheviour / SpermCompetition
Other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NUCB1 Q9VVK7
Obp51a Q7KE33
Dmel\CG11977 Q9VHJ0
CG13382 Q8IQS7
Acp53C14c Q6IGA4
CG31680 Q8T971
Obp56e Q7K088
Dmel\CG5162 Q9VX69
regucalcin Q9VYR1
CG17022 Q9VLB4
Calr Q9U916
Semp1 Q9VJN9
Dmel\CG31419 Q9VEL4
lincRNA.26 M9NEQ6
CG11955 Q95SR0
SP Q4V3K7
OstDelta Q7K110
Dmel\CG10029 Q9VI96
Dmel\CG17575 A1Z957
Dmel\CG3097 Q9W478
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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
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intr Q9VAT6
Pdi X2JGP4
Hexo2 Q9W3C4
Qsox3 Q9VD62
Phm E2QCL8
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CG6071 Q8T4B0
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Alp4 Q24238
Acp53C14c Q6GUV0
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0
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−1
20ºC 24ºC 28ºC
B
SFPs' TRANSFER
Hexo2 Q9W3C4
BEST:GH19547 Q7K5N8
BG642163 Q3HKQ0
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lectin-29Ca Q6GUX9
Dmel\CG17097 E2QCS7
Swim Q95S79
Dmel\CG15117 Q7K173
gi21430910 Q86BL9
Dmel\CG34130 Q0KI07
Dmel\CG17472 Q9VIL5
Spn77Bb Q9VWB4
lectin-30A Q6GV06
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Pdi X2JGP4
Ovulin P10333
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Fkbp14 Q9XZ54
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lectin-21Ca Q9VPS2
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GCS2alpha Q7KMM4
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CG17022 Q9VLB4
SP167 Q9VP92
Dmel\CG9806 Q9W2S8
SP Q4V3K7
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−0.5
−1
20ºC 24ºC 28ºC
SPnetwork
OvulinNetwork
PostMating Baheviour / SpermCompetition
Other