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**Synchrony of gamete release and sperm competition in
Arctic charr (*Salvelinus alpinus*)**

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synchrony, sperm competition

25 **Abstract**

26 A mismatch in timing between the release of male and female gametes in external
27 fertilizers may lead to failed fertilization or, under sperm competition, reduced
28 paternity. To quantify the actual synchrony of gamete release in a naturally
29 spawning population of fish, we placed video cameras on two spawning grounds of a
30 wild population of Arctic charr (*Salvelinus alpinus*). We captured 45 spawning events;
31 20 single male spawning events and 25 with more than one male participating, i.e.,
32 sperm competition. Together these spawning events included 85 ejaculations and
33 76.5% of these were released in sperm competition. The mean number of males
34 releasing milt in each spawning was 2.6. In sperm competition, guarding males
35 spawned more in synchrony with females than the subsequent sneaker males. Yet,
36 when males spawned alone with the female, sneaker males released their gametes
37 more in synchrony with females than guarding males. Our results provide essential
38 information for disentangling the importance of spawning synchrony and sperm
39 traits for fitness in an external fertilizer exposed to high levels of sperm competition,
40 and suggest a strong influence of male behaviour on female reproduction.

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49 **Introduction**

50 Sperm competition occurs when sperm from different males interact before
51 the fertilization of ova (Parker 1970; Birkhead & Møller 1998). In species where
52 sperm competition is common and little cryptic female choice is exerted, i.e., in
53 external fertilizers, there is strong intrasexual, selection on sperm traits (Gage et al.
54 2004; Hoysak & Liley 2001; Stoltz & Neff 2006; reviewed in Birkhead & Møller 1998;
55 Taborsky 1998). Here, mechanisms allowing more resources to be allocated to sperm
56 production should be selected among subordinate males as subordinates are more
57 likely to experience sperm competition than dominant males (Parker 1998). Thus, to
58 compensate for a disfavoured mating position, subordinate males should be selected
59 for increasing sperm number and velocity, but with a trade off against sperm
60 longevity (Parker 1993). Traditionally sperm numbers has been thought to be the
61 most important parameter for paternity in external fertilizers (Peterson & Warner
62 1998), however, recent work suggests that sperm velocity, i.e., sperm swimming
63 speed, may also have a substantial impact. For example, Skjæråsen and colleagues
64 (2009) found that sperm velocity had the strongest impact on male fertilization
65 success in cod (*Gadus morhua*) and in Arctic charr (*Salvelinus alpinus*), the velocity of
66 a male`s sperm, relative to the velocity of the competing male`s sperm, is the best
67 predictor of a male fertilization success (Liljedal et al. 2008) Thus, both sperm
68 numbers and sperm velocity might be of importance for male fitness in sperm
69 competition.

70 In externally fertilizing fish species exposed to sperm competition, spawning
71 behaviour elicited by one individual may motivate the other to a behavioural

72 counter-response (de Gaudemar & Beall 1999; de Gaudemar & Beall 2000; Satou et.
73 al 1991). The timing of gamete release is important (Mjølnerød et al. 1998; Yeates et
74 al. 2007) as the release of sperm must be timed to fit within the time window of
75 receptiveness of the eggs. A mismatch between the release of male and female
76 gametes may lead to reduced or failed fertilization, or reduced paternity share under
77 sperm competition. It has been shown experimentally that asymmetry in sperm
78 release can have significant consequences for male fertilization success (Yeates et al.
79 2007). Accordingly, there has been selection for behaviour(s) to synchronize gamete
80 release in natural spawnings. However, no studies exist that measure the possible
81 differences, in gamete release synchrony, between individuals.

82 The Arctic charr has a lek-like mating system characterized by sperm
83 competition and alternative reproductive tactics among males (Fabricius 1953;
84 Fabricius & Gustavson 1954; Sigurjónsdóttir & Gunnarson 1989). During the
85 spawning season males aggregate at distinct lek sites (Skarstein & Folstad 1996;
86 Liljedal et al. 1999; Liljedal & Folstad 2003; Figenschou et al. 2004) and when the
87 sexually mature females arrive, males compete intensely over positioning and
88 fertilization opportunities. That is, when a female arrive, one male try to guard the
89 female from other males by aggressively chasing and biting other males approaching
90 the female (pers. obs.). Male's court females by gliding alongside her while quivering
91 with high frequency low amplitude waves (Fabricius 1953; Sigurjónsdóttir &
92 Gunnarson 1989; pers. obs.). Occasionally this type of stimulating behaviour from
93 the male(s) leads to female egg release (Fabricius 1953) and thus fertilization
94 opportunities (for both dominant and subordinate males; pers. obs.). That is, during

95 spawning, the nearby often smaller and less aggressive males regularly dart into the
96 spawning site that offers no protection against sneakers, and release their milt.
97 Sperm production and sperm velocity seem to be a very plastic trait in charr
98 (Rudolfson et al. 2006; Serrano et al. 2006; Haugland et al. 2009) and subordinates
99 are found to have higher initial sperm production and higher velocity than dominant
100 males (Rudolfson et al. 2006).

101 Dominant (hereafter termed guarding) and subordinate (hereafter termed
102 sneaker) males may differ in spawning synchrony of gamete release with the female
103 and under sperm competition the relative time difference in point of milt release
104 between the guarding and the sneaker(s) males may affect paternity. However,
105 empiric studies of the actual spawning event are to a large extent missing. By using
106 underwater video recordings in charr`s natural spawning environment, we estimated
107 the synchrony of gamete release between the female and the male, and the time
108 delay experienced by sneaker males.

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118 **Methods**

119 *Study site and video recordings*

120 The study was conducted during the spawning season (mid September, 2006
121 and 2007) at Lake Fjellfrøstvatn, located at 69°N, 125 metres above sea level in
122 Troms, northern Norway. The depth of the spawning grounds varies between 0.5
123 and 2 metres (Figenschou et al. 2004) and the bottom substrate consists mostly of a
124 thin layer of mud and algae over gravel and rocks (own observation). The recordings
125 were conducted using two Sony Handycam video cameras, equipped with Sony wide
126 conversion lenses (x0.6), placed in underwater housings, in addition to a watertight
127 Oregon Scientific (ATC-2K) action camera. The video cameras were mounted on
128 tripods and aimed at stationary females. To reduce human induced interference at
129 the spawning grounds, the cameras were left undisturbed for the duration of the
130 recording time (approx. 90 min.). Recordings from the Sony Handycam were filed on
131 Mini DV tapes using a long play setting, and later copied to DVD`s, whereas
132 recordings from the Action camera were stored on a memory card before
133 downloaded to a computer hard drive. The recordings were later analyzed using
134 Final Cut Express HD v3.0 (Copyright © 2002-2005, Apple Computer, Inc.), iMovie HD
135 v6.0.3 (Copyright © 1999-2006, Apple Computer Inc.) and Apple DVD Player v5.0.3
136 (Copyright © 2001-2008, Apple Inc.).

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138 *The spawning event and its definitions*

139 In total we recorded 69 hours and 40 minutes of Arctic charr spawning
140 behaviour and captured a total of 45 spawning events. 25 of the recorded events
141 were captured in 2006 and the remaining 20 events, in 2007. The actual female egg
142 release in a spawning event is often difficult to observe and in brown trout (*Salmo*
143 *trutta*) females may “fake” spawning (Petersson & Jarvi 2001). We defined a
144 spawning to have occurred when at least 6 different types of spawning behaviours
145 had taken place. We adapted the following specifications from Fabricius 1953;
146 Fabricius & Gustavson 1954; Sigurjónsdóttir & Gunnarson 1989 and Satou et al.
147 1991; Fleming 1996):

- 148 1. The female lay close to the bottom substrate with an erected anal fin and the
149 anterior part of the body pointing upwards (anchoring).

- 150 2. The female is courted by a male approaching from the back, gliding alongside
151 the female while quivering. Both male and female can be the first to quiver.
152 3. The quivering gets stronger and both male and female gape and quiver
153 vigorously. At this point the male and female genital tract is close to one
154 another and both presumably release their gametes. Released milt can be
155 observed as “clouds” in the water at this stage of the spawning.
156 4. Both the male and female propels forward, away from the chosen site. The
157 male frequently holds a higher velocity and thus leave the female behind.
158 5. Both individuals swim slightly upwards - from the substrate with the head still
159 pointing upwards.
160 6. The female returns to the spawning site in a short radius circle, probably to
161 protect and/or cover the spawned eggs.

162

163 *Male density, sperm competition and gamete release*

164 By counting the number of males that, at the actual spawning event, had
165 some part of its body within the radius of approximately 25 cm (i.e., one fish length)
166 from the female, we estimated male density at different time intervals between -2
167 and 4 sec away from female gamete release (see Figure 1). Moreover, it is
168 convenient to divide the level of sperm competition into two measurable categories
169 (Parker et al 1996). (i) Risk of sperm competition, which is the probability of a male
170 spawning with at least one other competing male, and (ii) intensity of sperm
171 competition, which is the number of males competing in a spawning event.

172 Male milt release and female egg release was identified to occur when
173 individual’s mouth was fully opened (gaping). Sperm competition was defined to
174 have occurred when more than one male, in the same spawning event, followed the
175 definitions of spawning behaviours. Asymmetry in male milt release was estimated
176 by counting the number of video frames, between the first and the subsequent
177 males gaping, using number of frames / frames per second. Due to uncertainty of
178 when the mouth was fully opened in some spawning events, the best time resolution
179 obtained was 0.1 sec.

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182 *Guarding and sneaking tactics*

183 Males may either invest in access to mates and eggs, i.e., fighting and chase
184 away other males, or save this effort and rather exploit the guarding male's
185 reproductive investments by sneaking reproduction quickly or inconspicuously
186 (Taborsky 2001). In the examined population, the male-male interactions
187 are vigorous and mating tactic seems to be size dependant (see Figenschou et al.
188 2004; Rudolfsen et al. 2006). It is easy to identify guarding and sneaking males during
189 pre-spawning behaviour. That is, in all the video-captured spawning events there
190 were initially one guarding male present, trying to defend the female and her chosen
191 site from other males. The competing males, which either; (i) dart into the spawning
192 site and released their milt in competition with other males, or (ii) stimulate the
193 female to spawn without the presence of the guarding male, were defined as
194 sneaker reproductive roles.

195

196 *Spawning synchrony*

197 By defining the moment of gaping as the time of gamete release (see Fleming
198 1996; Fitzpatrick et al. 2008), we estimated the synchrony between female and the
199 male gamete release. However, in some of the spawning events it was difficult to get
200 exact measurements as individuals sometimes spawn with their head pointing away
201 from the video camera or with one individual masking another. We were therefore
202 only able to estimate spawning synchrony in 34 out of the 45 recorded spawning
203 events.

204

205 *Statistical analysis*

206 To avoid influencing the natural behaviour in the examined populations, we
207 did not tag the individuals. Thus, the possibility of pseudoreplication is present in all
208 observations, especially from observations of sneaker males as they were difficult to
209 distinguish. In our analysis, we therefore choose statistically to treat each spawning
210 event from the female's perspective and we consequently treated each spawning
211 event as one independent data point. Although sneakers (by their nature) mate
212 opportunistically, we registered that females spawned with sneaker males without
213 the presence of a guarding male. Thus, we examined if spawning females ($N = 17$)

214 varied in their “preferences” for male mating tactic, respectively guarding or
215 sneaking, with a logistic regression (binomial distribution and logit function).
216 Moreover, we measured if there were any differences in female “preference” for
217 single versus multi-males spawning events (the latter hereafter termed sperm
218 competition). However, we did not observe any differences in female mating
219 preferences for neither male reproductive tactic, nor single spawning events or
220 sperm competition spawning events (data not shown, $P > 0.08$). Consequently, in the
221 recorded spawning events ($N = 45$), statistical significance in frequencies was tested
222 by Chi-square (χ^2) tests. Difference in spawning synchrony were evaluated with
223 nonparametric tests when (i) we had low sample size, (ii) the variables did not fulfil
224 the assumption of the homogeneous variances (Levene's test), or (iii): if the variables
225 not showed a normal distribution of residuals. However, estimates of spawning
226 synchrony between female and male's gamete release differ from estimates of
227 asymmetry in male milt release as sample size differ depending on the statistical
228 approach. Moreover, we were not able to sample all variables for all events, so
229 samples sizes vary throughout. Spawning synchrony in sperm competition, between
230 the guarding and the 1st sneaker male, was tested with one sample t-test as the
231 variables fulfilled the assumption for parametric tests. However, to improve the
232 visualization of the results, untransformed values are used in all figures. Finally, we
233 used STATISTICA 7.0 (Stat Soft, Inc. Tulsa, USA) and StatView for Windows 5.0.1, for
234 all the statistical procedures.

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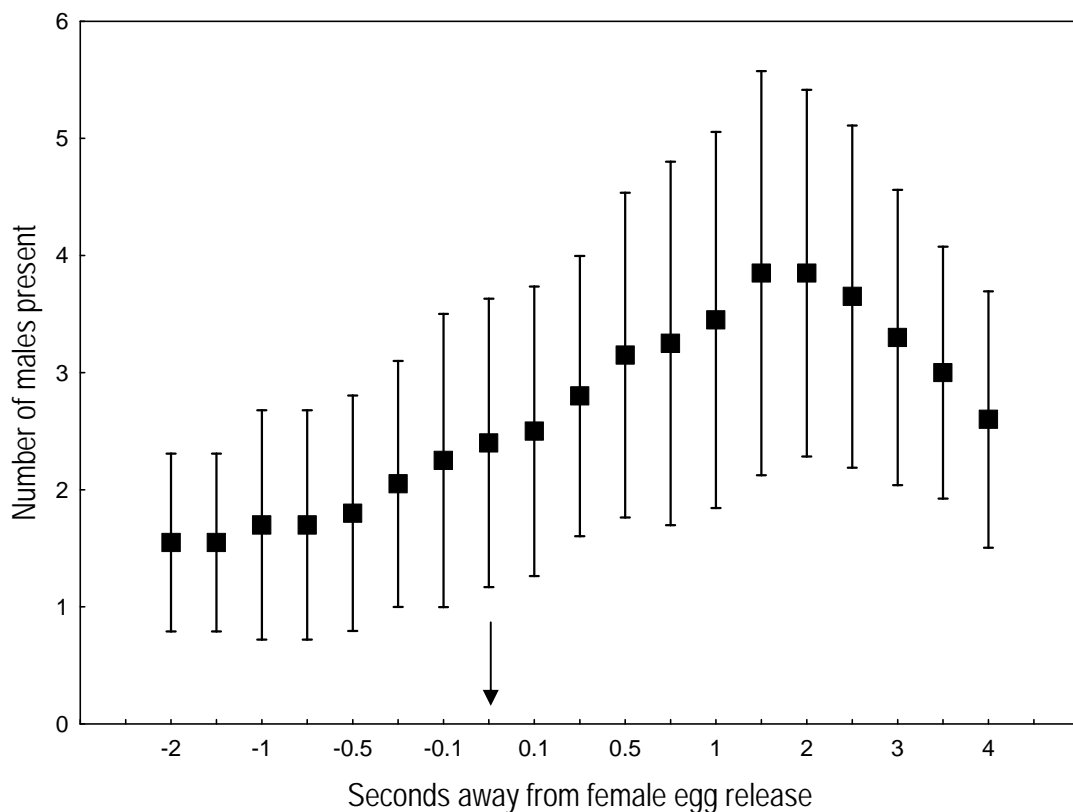
236 **Results**

237 *Male density at the spawning site*

238 Right before and during the spawning events there was an influx of males
239 towards the spawning site. That is, the number of males ranged from 0 to 9, with a
240 peak in male density 2 sec after female egg release (Figure 1). Between -0.25 and -
241 0.1 seconds before female egg release, an increase in mean male density was
242 observed (Wilcoxon signed-ranks test: $T = 7.5$, $N = 44$, $P < 0.001$; see Figure 1). The

243 mean number of surrounding males, at the moment of female egg release, was 2.89
244 ± 1.4 (mean \pm SD, range = 1 – 8), whereas male density 2.0 seconds after female egg
245 release was 4.59 ± 1.69 (mean \pm SD, range = 1 – 9). Males released milt from -0.15
246 before female egg release to 1.9 seconds after female egg release. During this period
247 ($\approx -0.1 - 2$ seconds) there was a significant mean increase of 1.78 male(s) around the
248 female (Wilcoxon signed-ranks test: $T = 5.5$, $N = 44$, $P < 0.001$).

249



250

251 **Figure 1**

252 The mean number (\pm SD) of males before, during and after female egg release ($N = 44$
253 spawning events). Black arrow (at 0.00 seconds at X-axis) indicates point of female
254 egg release.

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258 *Intensity and risk of sperm competition*

259 Commonly, the level of sperm competition is measured as either intensity (number
260 of males releasing milt), or risk (probability of experiencing sperm competition). In
261 the 25 sperm competition events observed, mean number of males releasing milt
262 was 2.6 ± 0.7 (mean \pm SD, range = 2 – 4, $N = 65$). When including the captured single
263 male spawning events (all 45 recorded events), mean number of males releasing milt
264 decreased to 1.88 ± 0.96 (mean \pm SD, range = 1 - 4, $N = 85$).

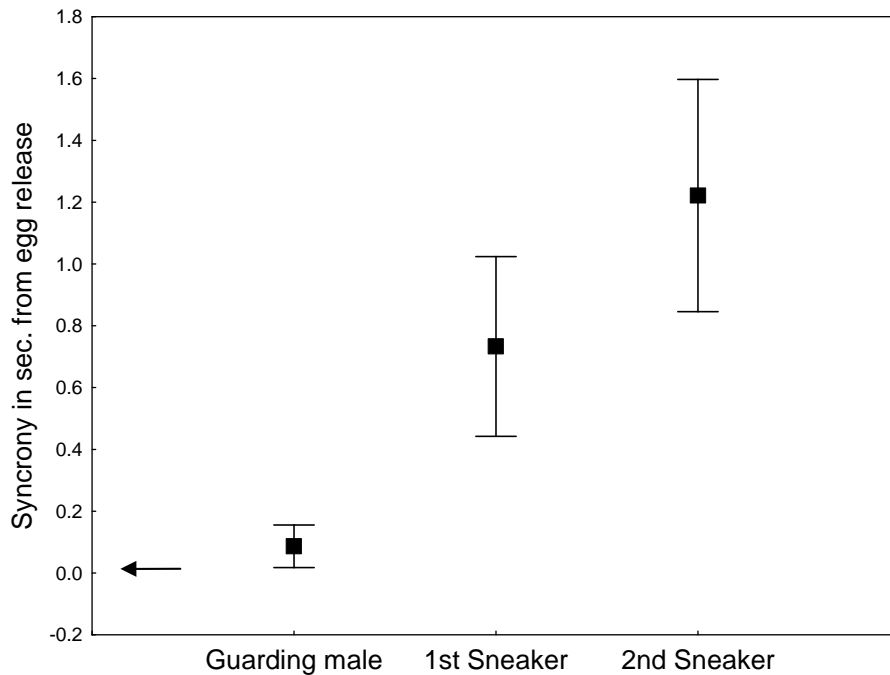
265 There was sperm competition in 25 (55.6%) out of 45 spawning events, suggesting
266 that spawning events with more than one male present, were not significantly more
267 frequent than single male spawning events ($\chi^2_1 = 0.56$, $P < 0.46$). In total we
268 registered 85 male milt releases during the 45 captured events. 65 of these were
269 released in sperm competition (76.5%) and 20 in single male spawning events. That
270 is, more ejaculates were released in sperm competition than in single spawning
271 events ($\chi^2_1 = 23.82$, $P < 0.001$). Guarding and sneaker males did not differ in risk of
272 sperm competition (Pearson's Chi-squared test, $\chi^2_1 = 2.45$, $P = 0.117$). Therefore, 42
273 out of 51 sneaker male ejaculates (82.4%) experienced sperm competition,
274 compared to 23 out of 34 guarding male ejaculates (67.6%).

275

276 *Gamete synchrony, sperm competition and different male tactics*

277 In all 25 sperm competition events, we measured the time delay in milt release
278 between the guarding male and the 1st sneaker, and it was on average ejaculating
279 0.48 sec (SE \pm 0.10) after the guarding male (one sample t -test, $t_{24} = 4.76$, $P <$
280 0.0001). However, by pooling the time delay estimates from all sneaker males, from
281 that of the guarding male, the overall mean delay for the "average" sneaker was

282 0.68 sec ($N = 40$). In sperm competition events, the guarding males ejaculated before
283 the sneaker males in 22 out of 25 events (88%) and there was a significant difference
284 in synchrony in milt release between the guarding male, 1st sneaker and 2nd sneaker
285 (Friedman ANOVA $\lambda^2 = 13.56$, $df = 2$, $P = .00114$, $N = 7$, see Figure 2).
286



287

288 **Figure 2**

289 Guarding and sneaker males milt release under sperm competition relative to female
290 egg release. Black arrow (at 0.00 seconds at Y-axis) indicates point of female egg
291 release. Sample sizes differ among tactic groups. Whisker denotes 0.95 confidence
292 intervals.

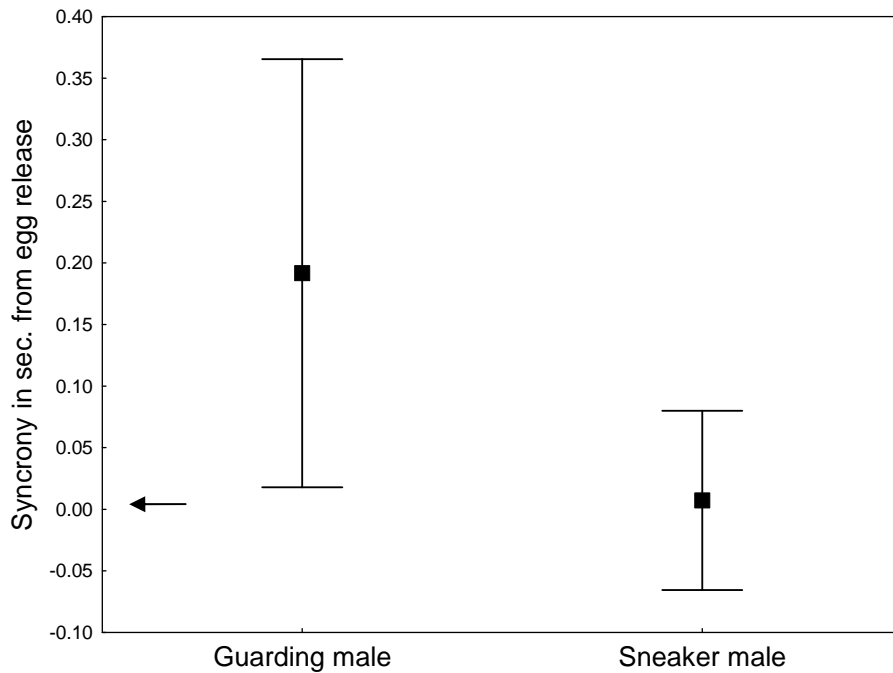
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294 *Female preference and gamete synchrony in single male spawnings*

295 In 9 of the 20 single male spawning events, females surprisingly spawn with sneaker
296 males (i.e., 45%; $\chi^2_1 = 2.00$, $P < 0.65$), suggesting no difference in female
297 “preference” for either guarding or sneaking males, i.e., ejaculates. Moreover, in
298 these single male spawning events, sneaker males milt release was significantly more

299 in synchrony with female egg release, than those of the guarding males (Figure 3;
300 Mann-Whitney U Test, $U = 4.0$, $P = 0.014$; mean = $0.191 \text{ SD} \pm 0.165$ ($N = 6$) and 0.007
301 $\text{SD} \pm 0.079$ ($N = 7$) sec. for guarding and sneaker males, respectively).

302



303

304 **Figure 3**

305 Guarding and sneaker males milt release in single male spawning events relative to
306 female egg release. Black arrow (at 0.00 seconds at Y-axis) indicates point of female
307 egg release. Whisker denotes 0.95 confidence intervals.

308

309 *Spawning initiated by guarding versus sneaker males*

310 Guarding males naturally courted females more often than sneaker males ($\chi^2_1 =$
311 428.5 , $P < 0.001$) and we observed that females spawned when being courted by
312 guarding males in 33 out of 45 spawning events (73.3%), which is significantly more
313 frequent than when being courted by sneaker males ($\chi^2_1 = 9.8$, $P = 0.002$).

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315

316 **Discussion**

317 We observed a high level of synchrony in gamete release between courting
318 males and females; with females normally releasing eggs first, quickly followed by
319 milt release by the guarding male, and subsequently the sneaker male(s). No
320 difference was observed in the risk of sperm competition between guarding and
321 sneaker males. However, in single male spawning events, sneaker males were more
322 in synchrony with female egg release than guarding males.

323 Females spawn when courted by guarding males in 33 out of the 45 captured
324 spawning events. In roach (*Rutilus rutilus*), which has a similar mating system to that
325 of charr, females differ in preferences for males (Wedekind 1996) and in salmonids it
326 is shown that male's dominance and aggression positively correlate with
327 reproductive success (Garner et al. 2009). This latter may be caused by females
328 releasing more eggs when courted by large males (de Gaudemar et al. 2000).
329 Females may also gain fitness by spawning with the most dominant males (Pettersson
330 & Järvi 1997; but see Reichard et al. 2007). This seems, however, not to be the case
331 in charr, where sperm from dominant males, in split-brood trials, not result in higher
332 quality larvae than sperm from subordinate males (Figenschou et al. 2007).
333 Moreover, male harassment and aggression in external fertilizers might limit the
334 effect of female choice. Studies on both brown trout and chinook salmon
335 (*Oncorhynchus tshawytscha*) show that females exhibit mate choice, but that choice
336 may be overruled by male-male competition (Pettersson et al. 1999; Garner et al.
337 2009). Yet, in the latter study, females also directed their aggression towards MHC-
338 similar males, providing a possible mechanism of female MHC choice in salmonids
339 (Garner et al. 2009).

340 Females should be choosy about when to release eggs to ensure that males
341 of high quality, with high quality ejaculates, fertilize her eggs. However,
342 approximately 70% of the released ejaculates were released in sperm competition,
343 with little female aggression directed towards males. Also in roach, females show no
344 resistance against multi-male fertilizations (Wedekind 1996). Thus, females may
345 increase fitness by spawning when the probability of multiple males fertilizing the
346 eggs is high, resulting in higher genetic variation in offspring (Jennions & Petrie 2000;
347 Reichard et al. 2007).

348 From a male perspective there was a higher risk of experiencing sperm
349 competition than avoiding it. An average of 2.6 ejaculates was competing in the
350 sperm competition events, whereas the overall mean decreases to 1.9 when also
351 including the single male spawning events. According to theory (Parker et al. 1996),
352 males should allocate most resources to sperm production when two males
353 participate in a spawning event, and decrease resource investments away from this
354 number with increasing or decreasing number of competitors. In our study, the
355 mean number of males engaged in sperm competition is thus close to what would
356 favour the highest allocation of resources to sperm production.

357 Subordinate, i.e., sneaker, males should, everything else equal, experience
358 sperm competition more frequently than guarding males (Parker 1990). Sneaker
359 males in the present study released 49 ejaculates in total; 9 in single spawnings and
360 40 in sperm competition. Yet, there was no difference in the probability of
361 experiencing sperm competition between the two male reproductive tactics. Males
362 in a favored mating role should experience a loaded raffle and invest less sperm
363 production according to theory (Parker 1990; see Smith et al. 2009). Compared to

364 dominant males, subordinate male charr have higher initial sperm velocity and
365 higher sperm cell density (Rudolfson et al. 2006) and this difference is in accordance
366 with findings in other external fertilizers (Vladic & Järvi 2001; Gage et al. 2004;
367 Skjæråsen et al. 2009). Thus, a loaded raffle rather than the probability of sperm
368 competition seem to be the best explanation for the observed differences in sperm
369 investments between sneaker and guarding charr.

370 Although females usually were surrounded by a large number of competing
371 males, 20 single male spawning events were captured. These events occurred while
372 the other males, either the guarding or the sneaker males, were occupied with intra-
373 sexual interactions that resulted in a delayed darting into the spawning site and no
374 milt release. Premature male milt release may result from misinterpretation of
375 female signals (Petersson & Järvi 2001) or, alternatively, from differences in risk
376 assessment between sneaker and guarding males. That is, sneaker males may take
377 greater risks than guarding males and sometimes release milt without females
378 releasing eggs (own observations). We did, however, not find any difference among
379 the number of guarding and sneaker males that released milt before females
380 released eggs. Yet, when males spawned alone with the female, sneaker males
381 released their gametes more in synchrony with females than guarding males.
382 Releasing milt just before the female releases eggs may result in an increased share
383 of paternity as the eggs will pass through a “cloud” of milt in the water (Fitzpatrick &
384 Liley 2008). In sum, sneaker male’s lack of synchrony in sperm competition events
385 seems not caused by sneaker male’s lack of ability to synchronise, but rather caused
386 by the guarding male’s mate guarding, preventing sneakers from gamete release
387 synchrony.

388 A male`s paternity share in sperm competition has previously been estimated
389 from his rank order and proximity to the female at spawning (Schroder 1982; Gross
390 1985; Fleming & Gross 1994; Fleming et al. 1996; Jacob et al. 2009). We observed an
391 increased number of sneaker males in the immediate proximity of the female and
392 the guarding male, immediately before egg release. This suggest that sneaker males,
393 ahead of time, are able identify, probably from the female and the guarding male`s
394 behaviour, that spawning will occur. This facilitates positioning among sneakers and
395 may thus explain the relatively low delay in sneakers milt release. That is, in sperm
396 competition, the guarding males released gametes more synchronous with the
397 female compared to sneaker males, but the mean time delay in milt release from the
398 guarding to the average sneaker was only 0.68 sec (see Table 3). For external
399 fertilizers, sperm of the first male are believed to have precedence over sperm of
400 males attending later in the same spawning event. For example, in Atlantic salmon, a
401 2.0 sec delay in sperm release caused significant reductions in paternity, with second
402 males achieving only 30% fertilization success, against an expected 50% (Yeates et al.
403 2007). However, for a species spawning in still water, a 0.68 sec delay in sperm
404 release among sneakers may possibly be compensated for by the sneakers higher
405 initial sperm velocity and their higher sperm numbers (Rudolfson et al. 2006).

406 The captured spawning events indicate that the actual spawning site seems
407 to be largely under female control, implying opportunities for female choice.
408 However, as sperm competition is common both for guarding and sneaker males;
409 and as synchrony in gamete release are high, also for sneakers, female mate choice
410 seems to be strongly influenced by male-male competition. This may have set the
411 stage for the evolution of the observed reproductive plasticity in charr.

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