

1 **Single-Cell Transcriptomics reveals relaxed evolutionary constraint of spermatogenesis in**
2 **two passerine birds as compared to mammals**

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25 **Abstract**

26 Spermatogenesis is a complex process where spermatogonia develop into haploid, mobile
27 sperm cells. The genes guiding this process are subject to an evolutionary trade-off between
28 preserving basic functions of sperm while acquiring new traits ensuring advantages in
29 competition over fertilization of female gametes. In species with XY sex chromosomes, the
30 outcome of this trade-off is found to vary across the stages of spermatogenesis but remains
31 unexplored for species with ZW sex chromosomes. Here we characterize avian
32 spermatogenesis at single cell resolution from testis of collared and pied flycatchers. We
33 find evidence for relaxed evolutionary constraint of genes expressed in spermatocyte cells
34 going through meiosis. An overrepresentation of Z-linked differentially expressed genes
35 between the two species at this stage suggests that this relaxed constraint is associated with
36 the lack of sex-chromosome silencing during meiosis. We conclude that the high throughput
37 of bird spermatogenesis, at least partly, is explained by relaxed developmental constraint.

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49 Introduction

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51 Spermatogenesis, the biological process by which haploid sperm cells are created from
52 diploid spermatogonia, is a highly complex process where at least seven somatic cell types
53 and 26 morphologically distinct germ cell classes are involved¹. Major chromatin remodeling
54 occurs during spermatogenesis such as programmed double strand breaks, homologous
55 recombination, chromosome synapsis, chromatin packing and chromosome inactivation². As
56 a result, the testis, where these processes occur, has the most complex transcriptome
57 among all tissues including the brain^{3,4}. Some of this complexity may be explained by the
58 prevalent transcription occurring across large parts of the genome, which is thought to
59 happen either as a non-adaptive side-effect of chromatin remodeling or as a mechanism of
60 general “transcriptional scanning” that allow DNA repair^{3,5,6}. However, the testis also
61 harbors the highest number of tissue-specific expressed genes among all tissues in
62 mammals, birds and insects⁷⁻¹¹. A crucial question is to what extent genes with testis-
63 specific expression reflect evolutionary innovations and species-specific differences in
64 mating behaviors and sperm-egg interactions or are shared among divergent species and
65 evolutionary constrained through effects of purifying natural selection to keep the complex
66 process of spermatogenesis functional.

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68 Analyses on the protein-coding sequences and transcription patterns show that testis-
69 specific genes are among the most rapidly evolving genes^{12,13} and that de-novo genes often
70 appear in the testis¹³. In addition, testis size^{14,15} and sperm morphology are astonishingly
71 diverse among animal species¹⁶. This fast evolution is thought to be largely driven by sexual

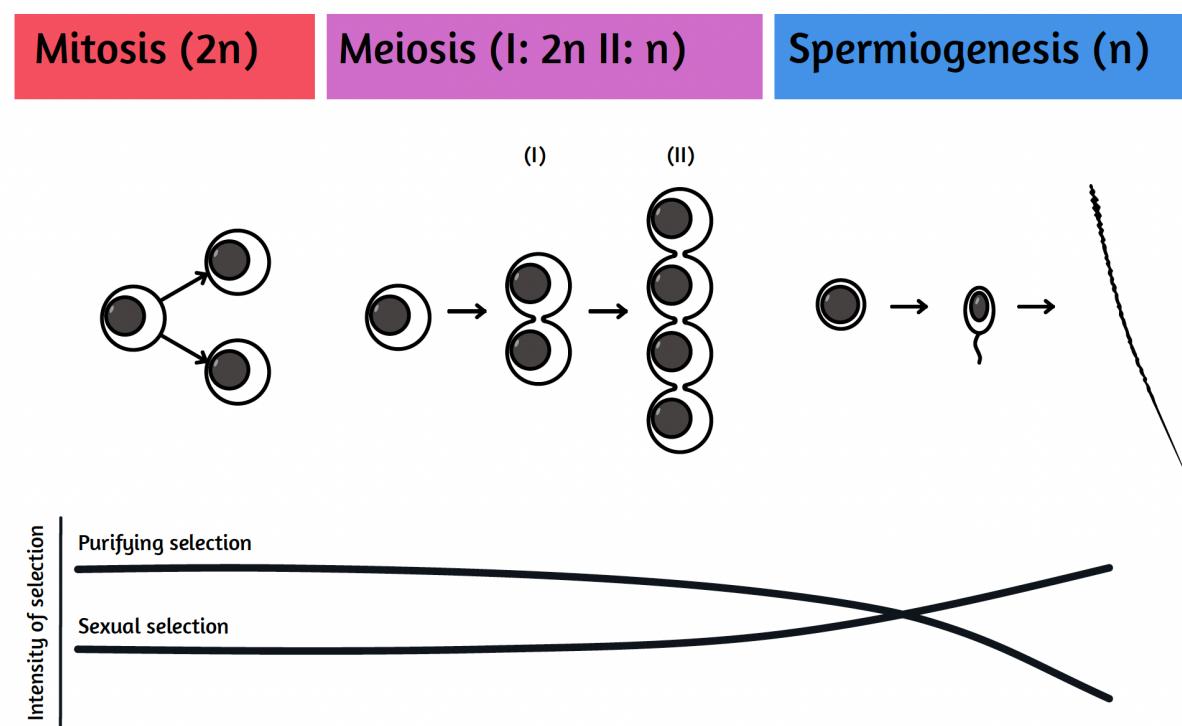
72 selection, mainly through sperm competition^{17–19} and cryptic female choice, the
73 mechanisms by which females select the outcome of sperm competition^{20–24}. Both these
74 processes are post-copulatory events that occur when females have mated with multiple
75 males, causing competition over fertilization success among ejaculates^{19,25}.

76

77 The great diversity in spermatogenesis and sperm phenotypes across animal species may
78 appear paradoxical in light of the central goal of this whole process being the same across
79 species, i.e. to ensure the transmission of genetic material to the next generation. To
80 achieve this goal, basic prerequisites always need to be fulfilled, including correct mitosis
81 and meiosis and finally maturation of sperm cells that ensures their motility and ability to
82 recognize and merge with the oocyte. Therefore the very basic processes of
83 spermatogenesis are similar among different animals and there is evidence that the
84 underlying genes are highly conserved^{26,27}. Thus, spermatogenesis is subject to a
85 combination of strong positive sexual selection through sperm competition and female
86 cryptic choice as well as strong purifying natural selection to maintain central functions.

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90 **Figure 1. Expected balance between positive sexual selection and purifying**

91 **natural selection across the different stages of spermatogenesis.**

92 Spermatogenesis can for simplicity be divided into three stages. The first stage includes
93 several types of spermatogonia, where some cells are in constant mitotic activity to preserve
94 the basal number of cells in the spermatogonia population while others start to mature and
95 get ready for the next step. The second stage includes all cells that are in the different stages
96 of meiosis also known as spermatocytes. The third stage includes the haploid spermatids
97 (round spermatids and elongating spermatids). At this final stage, genes coding for sperm
98 traits that will define characteristics such as swimming ability, velocity and compatibility
99 with female reproductive fluid and female gametes, are expressed. Stages one and two are
100 suggested to be subject of strict control ensuring the correct respective cell divisions that
101 keep the whole process functional. By contrast, purifying selection on genes acting during
102 the last stage of spermatogenesis is suggested to be relatively relaxed and sexual selection
103 acting on sperm traits that affect success in sperm competition and/or are favored by cryptic

104 *female choice to be strong.*

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106 The balance between positive sexual selection and purifying natural selection affecting DNA

107 sequences and gene expression is likely to differ between different stages of

108 spermatogenesis (Figure 1). Genes expressed during the two first stages affect basic cell

109 divisions and should be subject to strong purifying natural selection that prevents the

110 spread of detrimental mutations^{28,29} and the appearance of disruptive selfish elements such

111 as transposons³⁰ and meiotic drivers³¹. The first stage, where there is a continuous mitotic

112 proliferation of spermatogonia, is known to be very sensitive to mutations given the high

113 number of mitotic cell divisions³². This aligns with the presence of strong purifying selection

114 on both protein-coding genes and their regulatory elements that would benefit both

115 spermatogenesis in males and cell proliferation and oogenesis in females (Figure 1). The

116 genes underlying these early, basic stages in gamete production could potentially be subject

117 to sex-specific selection since male fitness relies on fast production of many gametes (i.e.,

118 stronger positive sexual selection) while female fitness is ensured through fewer gametes of

119 higher quality (i.e., stronger purifying selection). However, sex-specific gene regulation

120 provides resolutions to such intra-locus sexual conflicts by allowing the two sexes to express

121 different optimal phenotypes³³. While the mitotic divisions of the female germ cells only

122 occur in the embryonic gonads and then enter meiosis during fetal development and stay

123 arrested, mitotic proliferation continues postnatal in males and gametes are produced

124 throughout life.

125

126 Spermiogenesis, the final stage of spermatogenesis should be more species-specific than the

127 previous stages. We expect purifying selection on genes expressed during this final stage to

128 be relatively relaxed and sexual selection to be strong in the form of both sperm
129 competition and cryptic female choice³⁴ (Fig 1). Because strong sexual selection acting on
130 males may favor sperm traits in males that are detrimental for female fitness³⁵, inter-locus
131 sexual conflict could arise and speed up diversification^{36,37}. With the increased use of single-
132 cell RNA (scRNA) sequencing, we now have a better understanding of spermatogenesis,
133 especially in mammalian and insect model organisms, all of them having XY sex determining
134 systems^{13,38-42}. This has allowed the identification of major germ and somatic cell types in
135 the testis as well as the characterization of expression patterns throughout the different
136 stages of spermatogenesis. It has thereby also become possible to study evolutionary
137 aspects of spermatogenesis in greater detail. For example, comparisons between human,
138 macaque and mouse showed conserved gene expression of spermatogonia and more
139 diversification in the spermatid differentiation stage, which is consistent with relaxed
140 purifying selection and/or increased sexual selection acting toward the later stages of
141 spermatogenesis^{39,40}. Taken together, previous theoretical and empirical studies in
142 mammals and insects suggest faster adaptive evolution at the last stage of spermatogenesis.

143

144 The evolution of distinct male and female reproductive features are tightly intertwined with
145 the evolution of sex chromosomes. A striking characteristics of mammalian spermatogenesis
146 is that sex-chromosomes are inactivated during meiosis⁴³ and their transcription is
147 repressed at the post-meiotic stage³⁴. Comparisons between human, macaque and mouse
148 have revealed consistency in sex-chromosome silencing during meiosis (MSCI), except for
149 some escape genes in the case of primates^{39,40}. Apart from a few genes that remain
150 repressed post-meiotically, many X-linked testis-specific genes are highly expressed during
151 this very last stages of spermatogenesis (spermiogenesis). These genes evolve fast and tend

152 to vary also among closely related species and may often be underlying the evolution of
153 hybrid male sterility^{34,44}.

154

155 Bird spermatogenesis, where spermatogenesis happens in the homogametic sex, is still
156 poorly understood⁴⁵, especially in passerines and has only been described
157 morphologically^{45,46} or using bulk RNA sequencing¹¹. However, in order to understand the
158 different stages of spermatogenesis, cell diversity composition and underlying evolutionary
159 processes, a single cell approach is necessary^{10,38,42}. This is particularly important for
160 amniotes where the testis is composed of seminiferous tubules and multiple cell types and
161 cell stages are mixed⁴⁷.

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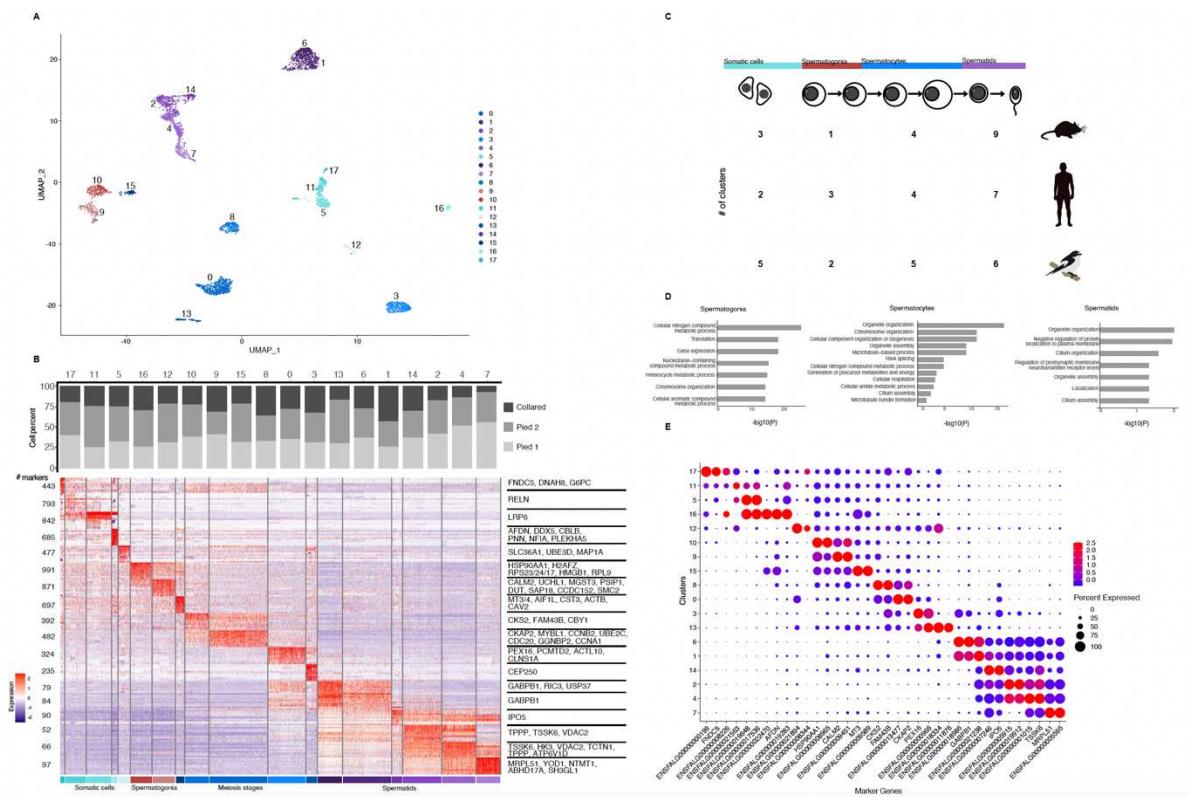
163 While the general expectations for the balance between evolutionary forces acting on genes
164 expressed during spermatogenesis are similar for birds and mammals, there may also be
165 some specific constraints derived from the different types of sex determination systems.

166 The expectation of relaxed purifying selection and increased sexual selection acting toward
167 the later stages of spermatogenesis^{39,40} is similar (Figure 1). However, since meiotic sex-
168 chromosome inactivation (MSCI) is triggered by the absence of homologous sex-
169 chromosome pairing partners in the heterogametic sex⁴⁸, it does not apply to
170 spermatogenesis in birds since males are the homogametic sex. Therefore, Z-linked testis-
171 specific genes may be active during all stages of spermatogenesis in birds.

172

173 In this study we present a detailed molecular characterization of passerine spermatogenesis
174 based on scRNA-seq of the testis of collared (*Ficedula albicollis*) and pied (*Ficedula*
175 *hipoleuca*) flycatchers with the aim to reveal conserved and species-specific evolutionary

176 features of this central process. The two closely related species of flycatchers are widely
177 used in studies on ecology and evolution⁴⁹ and male hybrids resulting from crosses between
178 the two species are known to experience impaired sperm production⁵⁰. There is also
179 evidence for post-mating, pre-zygotic isolation⁵¹. These findings imply divergence in genes
180 underlying spermatogenesis and make it possible for us to investigate conserved and
181 diverged evolutionary features on a relatively short evolutionary time scale. We investigate
182 the hypothesis of strongly conserved patterns of gene expression during the first stages of
183 spermatogenesis and diverged features at the final stage in a ZW sex-determining system.
184 Since our study is the first characterization of spermatogenesis at a single cell level for a ZW
185 sex-determining system, an additional major goal is to assess the activity of the Z
186 chromosome at the different stages of the process. We will also investigate whether Z-
187 linked, testis-specific genes are more conserved or diverged at the different stages of
188 spermatogenesis compared to testis-specific genes located on the autosomes.
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192 **Figure 2. Single cell RNA sequencing of testis samples from *Ficedula* flycatchers reveals 18**
193 **spermatogenesis cell types. (A) Visualization of consensus clustering of testis cells from 3**
194 **flycatcher individuals in UMAP (Uniform Manifold Approximation and Projection). (B)**
195 **Spermatogenesis cell cluster composition in terms of the percentage of cells from each testis**
196 **sample (i.e. individual bird) and heatmap showing the mean expression of each top 10**
197 **marker genes of the 18 cell types found. Representative makers with annotated function are**
198 **listed to the right and the total number of markers for each cluster are listed to the left side.**
199 **(C) Comparison of the number of clusters of testis cell types found in flycatchers, human and**
200 **mouse using a similar methodology (data from Hermann et al. 2018). (D) Representative GO**
201 **terms for the three main stages of spermatogenesis in flycatchers, with FDR-corrected p-**
202 **values shown for each GO term. (E) Subset of the best 2 or 3 markers specific to flycatchers**
203 **for each cluster, most of the markers lack functional annotation or do not have an ortholog**
204 **and therefore are shown with their Ensembl gene ID.**

205 **Results**

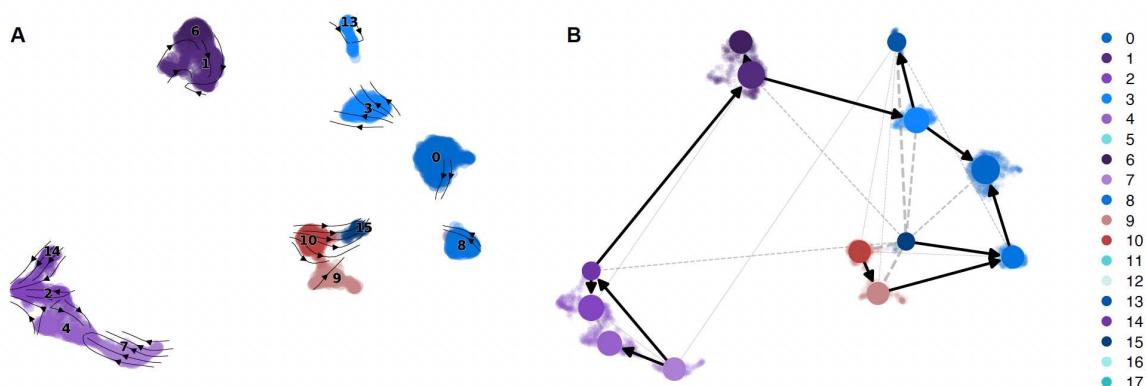
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207 **Characterization of bird spermatogenesis**

208 Using the 10X genomics Chromium Single cell platform, we generated scRNA data from
209 testis cell suspensions of two pied flycatchers and one collared flycatcher. After data pre-
210 processing and cleaning, we recovered 4936 cells across our three samples that resulted in a
211 consensus of 18 distinct clusters (Fig 2A) where each cluster contained cells from all three
212 individuals (Fig 2B). We identified gene markers for each cluster, and a subset of the gene
213 markers is shown in (Fig 2C, 2F) (Table S1). Most of the top cluster marker genes for
214 flycatchers are not functionally annotated or do not have a corresponding ortholog in
215 mammals. However, 65 representative flycatcher markers had unique orthologs in human
216 and we found three genes that are also highlighted as important markers of different stages
217 of spermatogenesis in mammals. A marker gene for undifferentiated spermatogonia,
218 UCHL1, a pachytene marker, MYBL1, and an elongating spermatid marker, TSSK6 were
219 found in mouse, human, macaque, and flycatchers. We also found several markers for
220 mammalian spermatogenesis cell types that were expressed in our clusters of flycatcher
221 spermatogenesis, (Fig S1, S2) and therefore were useful in the assessment of the identity of
222 most of our clusters. Two of our 18 clusters were characterized as belonging to the initial
223 stage of spermatogenesis (i.e. as spermatogonia), five to meiosis stages (i.e. spermatocytes)
224 and six to the final stages of differentiating spermatids. In addition, five clusters were
225 identified as somatic cells (Fig 2C). Among the markers indicative of clusters belonging to
226 the somatic cells, we found mammalian markers for macrophages, sertoli cells, leydig cells
227 and structural cells (Fig S2). Gene Ontology (GO) analysis based on the gene markers

228 showed relevant biological processes for all the three main stages of spermatogenesis (Fig
229 2D). Consistent with this functional identification of clusters, velocity analysis and the PAGA
230 graph obtained with ScVelo indicated a congruent order of the clusters with the main stages
231 already identified (Fig 3). A general comparison of all markers from the three main stages
232 with all the markers identified by Hermann et al., 2018 for mouse and humans, shows that
233 the differentiating spermatids have significantly less shared markers between mammals and
234 flycatchers than the first two stages (χ^2 -test with $df = 2$, $\chi^2 = 76.4$, p -value $< 2.2e^{-16}$) (Table
235 S2, S3) (Fig 4).

236



237

238 **Figure 3 Trajectory of *Ficedula flycatcher* testis cells.** (A) Velocity dynamics of germline cell
239 clusters. (B) PAGA graph of germline cell clusters, the arrows indicate connection strength
240 and possible direction of differentiation.

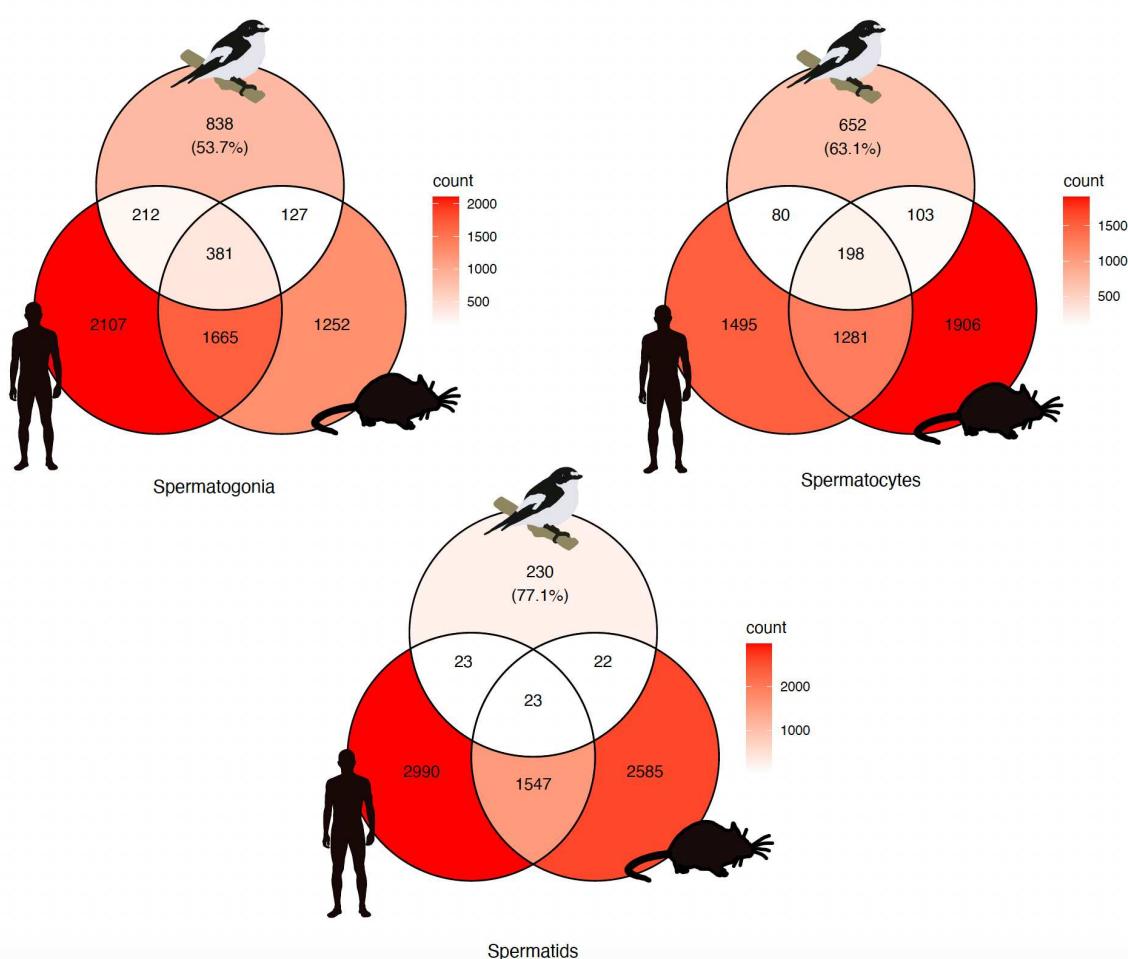
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242 Gene expression patterns

243 Excluding the somatic cell clusters and considering all expressed autosomal and Z-linked
244 genes, we found a similar mean expression across the cell clusters (Fig 5A) (Table S4). At this
245 last stage of spermatogenesis when spermatids are differentiating, the increase in variance

246 in gene expression is due to a very high expression of a few genes. Cluster 13 belonging to
247 the meiosis stages, is the only cluster showing a pronounced tendency for a higher average
248 expression of Z-linked genes with respect to the autosomal genes. When restricting the
249 analysis to the top 500 expressed genes, we still find the pattern described above for cluster
250 13, and that there is an increase in mean expression along the ordered timeline (Fig 5B).

251



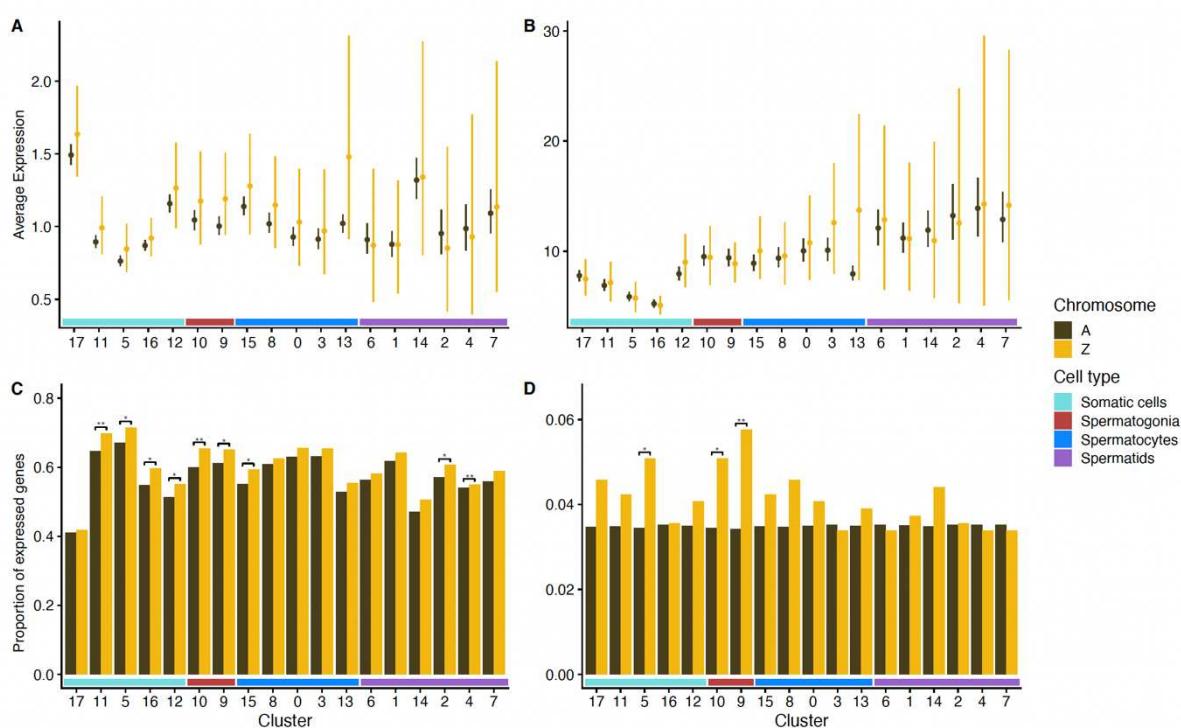
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253 **Figure 4. Venn diagrams for the three main stages of spermatogenesis showing shared**
254 **and private marker genes between flycatchers, human and mouse. The percentage in**
255 **brackets corresponds to the percentage of private flycatcher markers. Spermatids share the**
256 **least number of ortholog markers between flycatchers and either human or mouse as**
257 **compared to the other stages of spermatogenesis.**

258

259 We also tested for an enrichment of Z-linked genes among the expressed genes in each
260 cluster. When considering all genes with average expression > 0.01 , we found a general
261 trend of enrichment of Z-linked genes across all stages of spermatogenesis, but significant
262 enrichment of Z-linked genes was only found for genes expressed in four somatic clusters,
263 the two spermatogonia clusters, one meiosis cluster and two spermatid clusters (Fig 5C)
264 (Table S5). This finding remains consistent for the two spermatogonia clusters and one
265 somatic cluster when restricting the analysis to the top 500 genes (Fig 5D) (Table S6).

266



267

268 **Figure 5. Average expression and proportion of expressed genes across all 3 testis samples**
269 **from flycatchers for each cell clusters.** Average expression of all genes located on the
270 **autosomes or on the Z chromosome (A) For all expressed genes and (B) for the top 500**
271 **expressed autosomal and Z-linked genes. Error bars were obtained by bootstrapping. (C)**
272 **There is a significant enrichment of Z-linked genes expressed in four somatic clusters, two**

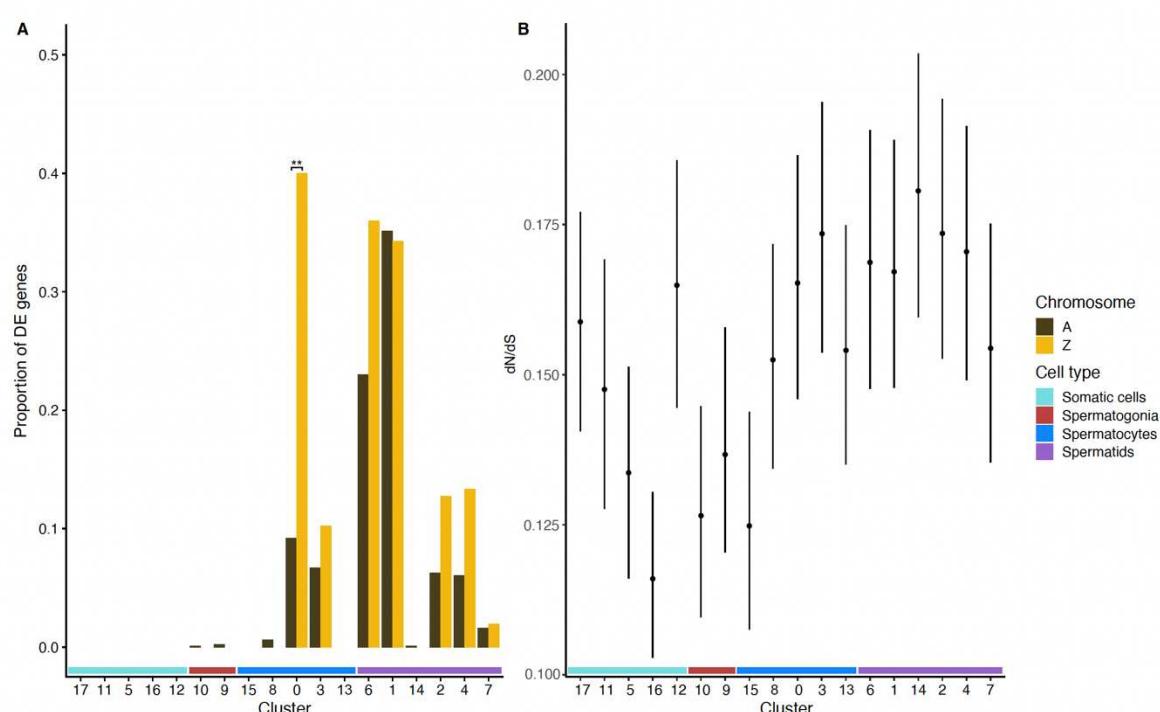
273 *spermatogonia clusters, one meiosis cluster and two spermatid clusters. For all genes with*
274 *expression level >0.01. (D) There is a significant enrichment of Z-linked genes among the top*
275 *500 genes expressed in one somatic cluster, and in two spermatogonia clusters.*

276

277

278 We calculated fold-change of gene expression for each cell cluster between the two
279 flycatcher species using Wilcoxon rank sum test for differential expression (DE) analysis (Fig
280 S3-S6) (Table S7). Very few or no genes were differentially expressed in the 5 clusters of
281 somatic cells and in the two clusters of spermatogonia cells. Most DE genes were found in
282 two clusters belonging to the meiosis stage and in five clusters belonging to the last stage of
283 spermatogenesis. Of these, cluster 1 had the most DE genes of all clusters (i.e. 291 DE
284 genes) (Fig 6A). Consistent with findings in species with XY sex determination, we thus find
285 most differences in gene expression between the two species towards the later stages of
286 spermatogenesis (generalized linear model with binomial distribution, 1.63 ± 0.07 , $Z =$
287 25.14 , $p\text{-value} < 2e^{-16}$) (Table S8). We tested for overrepresentation of Z-linked DE genes and
288 found that one of the cell clusters going through meiosis (i.e. cluster zero) had a significant
289 overrepresentation of DE genes on the Z chromosome (hypergeometric test, $p=0.006$)
290 (Table 1). We lack statistical power to test for overrepresentation of Z-linked genes in cell
291 clusters with few DE genes but most clusters with several DE genes show a tendency for
292 overrepresentation of Z-linked genes (Fig 6A). We found significant GO terms among the DE
293 genes in clusters belonging to meiosis and the final stage of spermatogenesis (i.e. cluster 0,
294 3, 6, 1, 2 and 4) (Table S9). These GO terms were mainly related to cellular respiration and
295 cell motility. Moreover, we found eleven DE genes located on the mitochondrial

296 chromosome, four autosomal genes that coded for mitochondrial proteins and two genes
297 on the Z chromosome that also coded for mitochondrial genes (Table 2).
298



299

300 **Figure 6. Patterns of differential gene expression between the two flycatcher species**
301 **among the different testis cell clusters and signatures of gene sequence evolution. (A)**
302 *Proportion of DE genes between collared and pied flycatchers in each cluster. DE genes are*
303 *only found in clusters belonging to spermatogenesis, most of them are present in the meiosis*
304 *clusters and the spermiogenesis clusters. There is a general tendency for overrepresentation*
305 *of Z-linked genes among the DE genes between the two species. However, only*
306 *spermatocytes of Cluster 0 belonging to the meiosis stage show a significant*
307 *overrepresentation of Z-linked genes. (B) d_N/d_S mean value for the top 500 expressed genes*
308 *by cluster. There is an increase in d_N/d_S mean value along the spermatogenesis timeline*
309 *consistent with a higher constraint at the beginning of the process and relaxed constraint*
310 *towards the end.*

311

312

313 **Signatures of gene sequence evolution across the stages of spermatogenesis**

314

315 The average d_N/d_S values between collared and pied flycatchers for the genes expressed in
316 the different cell clusters ranged from 0.1 to 0.2, which indicates that genes are on average
317 evolving under constraint across all stages of spermatogenesis. However, the values are
318 generally smaller, which is indicative of more constraint, for the somatic and for the first cell
319 clusters, while the d_N/d_S values increase towards the end of the timeline, which is indicative
320 of less constraint or an increasing contribution of positive selection (Figure 6B). These
321 findings are hence indicative of stronger constraint on the initial spermatogonia stages of
322 spermatogenesis and that the magnitude of purifying selection slightly relaxes toward the
323 end of the timeline, or that that there is positive selection acting on an increasing fraction of
324 sites within the genes that are expressed during the final stages of spermatogenesis.

325

326

327 **Discussion**

328

329 Functional spermatogenesis is a pre-requisite for male fertility in sexually reproducing
330 organisms, but has previously only been well characterized in a handful of model systems
331 such as *Drosophila* and a few mammalian species^{13,38–40,42}. In all these previously studied
332 model systems males constitute the heterogametic sex, implying that some major
333 evolutionary features are shared between them such as the regulatory constraints

334 associated with meiotic sex chromosome inactivation (MSCI) and post meiotic gene
335 repression, which select against sex-linked meiotic genes. Based on scRNA-seq of the testis
336 of two closely related passerine bird species, where males constitute the homogametic sex,
337 we can reveal both, striking evolutionary similarities and differences, compared to previous
338 findings based on the study systems with heterogametic males. One of our major goals was
339 to characterize the relative average expression of Z-linked genes to autosomal genes along
340 the different stages of spermatogenesis. We know from oogenesis in XY systems, which
341 happens in the homogametic sex, that there is an absence of MSCI given that there are no
342 unpaired chromosomes. However, before the moment of chromosome synapsis one of the
343 normally silenced X chromosomes needs to be unsilenced causing a hyperexpression of the
344 X chromosome (i.e. dosage decompensation)⁵². Birds have been found to have partial
345 dosage compensation regulated on a gene-by-gene basis^{53,54}. This is mainly evident in the
346 heterogametic females where the Z:A ratio has been found to be < 1 while homogametic
347 males have a Z-linked average expression similar to the autosomes (i.e. Z:A ~ 1).
348 Nevertheless, we observe a high average expression of the Z chromosome with respect to
349 the autosomes in one of the cell clusters belonging to meiosis (cluster 13; Fig 5A, 5B). This
350 signal appears very similar to the moment in mammal oogenesis when the X chromosome is
351 showing dosage decompensation during meiosis to enable the following chromosome
352 synapsis⁵². Since we know that some genes on the Z chromosome show dosage
353 compensation while others do not, it is possible that the signal we observe reflects
354 decompensation of the compensated genes similarly to what happens during mammalian
355 oogenesis.
356
357 In agreement with earlier studies investigating signals of molecular evolution across

358 different stages of spermatogenesis in mammals^{34,39,40,55}, we find that the first stage of
359 spermatogenesis shows several signs of strong evolutionary constraint. These signs include
360 higher conservation of gene expression between the two flycatcher species at this stage
361 compared to the later stages (Fig 6A), higher conservation of protein-coding sequences of
362 genes expressed at this stage (Fig 6B), and more detected shared orthologous gene markers
363 between flycatchers and mammals among the gene markers used to characterize the cell
364 clusters (Fig 4). Like in mammals, we also find a significant enrichment of Z-linked genes in
365 the two clusters of this stage⁵⁵. All together these results reaffirm the prediction that basic
366 processes such as mitotic cell division are highly conserved even among divergent taxa due
367 to strong purifying selection to keep functionality.

368

369 During the second stage of spermatogenesis when the different steps of meiosis occur, we
370 expected to find lesser constraint than the first stage of spermatogenesis. However, we find
371 evidence of even lesser constraint than expected based on earlier observations in XY
372 systems. Consistent with expectations, genes expressed in the second stage show lower
373 conservation of protein-coding sequences compared to genes expressed in the first stage of
374 spermatogenesis. However, two cell clusters belonging to the stages of meiosis contain an
375 unexpected high proportion of genes that are differentially expressed between the two
376 flycatcher species (Fig 6A). This result indicates that gene expression at this stage is less
377 constraint by purifying natural selection and/or by intra-locus sexual conflict as compared to
378 mammals. While this finding may be specific to the flycatcher system, given the absence of
379 MSCI during male meiosis in ZW systems it could also be shared by other ZW species. In
380 mammals, MSCI and post-meiotic repression of X-linked genes result in many testis-biased
381 genes having been copied from the X chromosome to the autosomes.⁵⁶⁻⁵⁸. Alternatively, X-

382 linked testis-biased genes are highly expressed in earlier stages of spermatogenesis and the
383 transcripts prevail until the final stage where its function is necessary⁵⁹. By contrast, testis-
384 biased genes can remain on the Z chromosome and be synthetized and expressed when
385 needed in birds. We indeed find a significant signal of enrichment of Z-linked genes among
386 the genes that were differentially expressed between the two flycatcher species during this
387 second stage of spermatogenesis. While this result again may be specific for the flycatcher
388 case, it is also consistent with relaxed constraint specifically associated with the absence of
389 MSCI which is a universal feature for birds and all ZW systems. Studies of additional species,
390 where spermatogenesis happens in the homogametic sex, are needed to provide a broader
391 picture. Nevertheless, since male fitness relies on fast production of many gametes, we
392 suggest that this relief of constraint may potentially explain why bird spermatogenesis is
393 four times faster compared to spermatogenesis in mammals.

394

395 As predicted, the third and last stage of spermatogenesis in the flycatchers shows even
396 more clear signs consistent with relaxed purifying natural selection and/or strong positive
397 sexual selection than the second stage. Protein-coding sequence divergence of genes
398 expressed in the last stage is on average highest among all stages and we find a significantly
399 higher proportion of DE genes between the two flycatcher species both in terms of number
400 of DE genes and in terms of number of cell clusters with DE genes. In agreement with what
401 has been described for mammals we also find more private markers at this stage and
402 overall, less genes, for which one-to-one orthologs to mammals could be identified. One
403 possible explanation for this observation is the presence of more de novo genes that are
404 species-specific at this stage, similar to what has been found among macaque, human and
405 mouse⁴⁰. During this last stage of spermatogenesis, when the sperm cells obtain traits that

406 influence swimming ability and other important functions known to affect success in sperm
407 competition and cryptic female choice, a relatively high degree of expression of sex
408 chromosome linked genes is expected³⁴. This is because sex chromosomes are in general
409 known to accumulate genes with sex-biased functions⁶⁰. Since the Z-chromosome spends
410 most of its time in males, the fixation of Z-linked mutations with male-biased fitness
411 functions are expected to be favored by positive sexual selection^{61–64}. There were
412 tendencies for enrichment of expressed Z-linked genes both among all the clusters at this
413 stage and among the DE genes between the two species of flycatchers. These findings are
414 consistent with evidence of an overall elevated sequence and expression divergence of Z-
415 linked genes compared to the autosomal genes in flycatchers^{11,65} and in other birds^{63,66}.
416 However, fast evolution of Z-linked genes can aside from positive sexual selection also be
417 caused by genetic drift in combination with a higher mutation rate in males^{67,68}. The
418 observed tendency for Z-chromosome enrichment of expression divergence associated with
419 the last stage of spermatogenesis could therefore also reflect relaxed purifying selection
420 instead of increased positive sexual selection. However, across the two last stages of
421 spermatogenesis, we find several significant GO terms for biological processes among the
422 DE genes between the two flycatcher species that are mainly related to cellular respiration
423 and cell motility. The presence of GO terms related to cell motility reinforces the idea that
424 sexual selection related to sperm performance has an important effect on the fast evolution
425 of genes coding for these traits at the final stage of spermatogenesis. Since F1 hybrids
426 resulting from crosses between collared and pied flycatchers experience hybrid dysfunction
427 in terms of severely reduced fertility⁵⁰ and in terms of elevated metabolic rate⁶⁹, our results
428 add to previous evidence suggesting that mito-nuclear incompatibilities may be causing or
429 contributing to such dysfunction.

430

431 There are some major similarities between XY and ZW systems in terms of the selection
432 pressures acting at the different stages of spermatogenesis with strong purifying selection
433 during the first stage of spermatogenesis and sexual selection or relaxed purifying selection
434 during the final stage. However, a major difference is the developmental constraint that
435 heterogametic males experience in XY systems that males in ZW systems lack. This lack of
436 constraint in theory allows the Z chromosome to keep testis-biased genes that also may
437 include genes with sexually antagonistic fitness effects. We detect signals of fast evolving Z-
438 linked genes expressed not only during the last stage of spermatogenesis but also already at
439 the second stage thereby revealing a possible key evolutionary difference between this
440 process in birds and mammals. We suggest that the high throughput of bird
441 spermatogenesis, which is four times faster compared to mammals⁷⁰, at least partly is
442 explained by relief of evolutionary constraint, possibly also connected to an advantage in
443 the intra-locus sexual conflict considering the optimal solution to the trade-off between
444 gamete number and quality. Our study is the first characterization of spermatogenesis at a
445 single cell level for a ZW system and it thereby opens the doors for future studies exploring
446 various aspects of spermatogenesis in ZW systems such as infertility, molecular evolution
447 and sex-chromosome evolution.

448

449

450 **Methods**

451 **Sample collection, cell suspension preparation and sequencing**

452

453 Two pied flycatcher (*Ficedula hypoleuca*) and one collared flycatcher (*Ficedula albicollis*)
454 males were sampled from the monitored populations on Öland (57°100N, 16°580E),
455 Sweden⁴⁹ in May of 2019. The individuals were trapped in nest boxes while defending
456 territories before nest building at the beginning of the breeding season. These birds were
457 briefly kept in outdoor aviaries and then transported in individual cages over night to our lab
458 facilities at Uppsala University. By choosing this timing during the breeding season, we
459 ensured that the individuals possessed fully functional testis, because the testis degenerates
460 once the reproductive season is over in this species. The animals were sacrificed and
461 immediately dissected. All animal handling was done following Swedish regulations with the
462 required permits approved.

463 The testes were placed in a cold petri dish with PBD BSA buffer, cleaned from any other
464 visible tissue cells and cut in half. A half of testis tissue was then put in 3ml of buffer PBD
465 BSA and dissociated by mechanical dissociation using the gentleMACS Dissociator (Miltenyi
466 Biotech, Bergisch Gladbach, Germany) with the gentleMACS C Tubes using the preset for
467 mouse spleen as recommended by the manufacturer. Next, the cell suspension was
468 centrifuged to assure all cells would get down the cap and the walls of the tube. Finally, the
469 cell suspension was homogenized by pipetting and filtered using a cell strainer of 70(μm).

470

471 After mechanical dissociation, an aliquote of the cell suspension was stained with propidium
472 iodide and Hoechst for live death cell staining, examined under the microscope, and cell
473 viability was estimated to be at least 80%. Cells were counted using a Neubauer chamber
474 and the cell suspension was then diluted to achieve the required concentration of 1 x 10⁶
475 cell/ml. The final cell suspension of a total volume of 500 ul was immediately delivered to
476 the sequencing platform for library preparation with 10X genomics Chromium Single Cell 3'

477 v3 kit for scRNAseq. The whole process between sacrificing the animals and handing in the
478 final cell suspension, lasted 2 to 3 hours during which the samples were kept on ice at all
479 times. The 3 libraries were sequenced in one NovaSeq SP flow cell, yielding an approximate
480 of 215 million reads per sample.

481

482 **Data processing and analysis**

483

484 We created a custom reference for cell counting using the 10x Genomics Cell Ranger v.
485 4.0.0.⁷¹ command mkref following default settings and the well documented CellRanger
486 pipeline. For that, we used the publicly available genome .fasta file (v. 1.4) of the collared
487 flycatcher and the collared flycatcher annotation .gtf file from *Ensembl* (v. 1.4). Count
488 matrixes for every gene in each individual cell for the three samples were obtained. Using
489 this output and the .gtf file, a .loom file was generated for each of the three samples using
490 the command run10x from the python package Velocyto v. 0.17.17⁷². Finally, this file was
491 exported to Seurat v. 3.2.0⁷³ for filtering, normalization and clustering (described below).

492

493 **Filtering, normalization and clustering**

494 The three .loom files were imported to R, transformed to Seurat objects and then merged to
495 a single object. We filtered out all cells with less than 200 features and more than 2500
496 features as well as cells with less than 5% of mitochondrial genes. The normalization was
497 done using anchors to integrate the 3 samples and SCTransform as documented in the
498 Seurat pipeline. Finally, we followed the standard Seurat workflow for clustering using
499 UMAP (Uniform Manifold Approximation and Projection). Because spermatogenesis is a
500 continuous non-discrete process, we obtained a big cluster containing a heterogenous cell

501 composition with no clear gene markers, therefore that cluster was excluded. The remaining
502 cells were re-clustered.

503

504 Marker genes for each cluster were identified using the Seurat function FindAllMarkers. The
505 default setting uses the Wilcoxon rank sum test and calculates average log fold change for
506 each gene between the clusters. We also found all markers using the ROC standard AUC
507 classifier method, most of which were the same as the markers found with Wilcoxon rank
508 sum test. We selected the top 3 best markers for each cluster having the highest AUC value
509 Figure 2 F.

510

511 **Characterization of the cell clusters**

512 We searched for the presence of all the previously identified markers for the different
513 spermatogenesis stages in human^{38,41}, mouse^{38,42} and macaque^{39,40} in our flycatcher cell
514 clusters. By crossing the information of the previously identified markers found in our data
515 we assigned identities to the cell clusters to the four major groups of cells found in
516 spermatogenesis: Spermatogonia cells, meiotic cells, differentiating spermatid cells and
517 somatic cells.

518

519 **Gene Ontology analysis**

520 We performed a Gene Ontology (GO) enrichment analysis for biological processes using the
521 webtool ShinyGO v.0741⁷⁴ with the collared flycatcher as a background. To remove
522 redundant and/or nested GO terms we used the web tool REVIGO⁷⁵. This analysis was done
523 per cluster using all identified markers and afterwards per cluster using all the differentially
524 expressed (DE) genes (see below).

525

526 **Velocity analysis**

527 To confirm the identity of the clusters with another analysis and infer the most likely time
528 trajectory, we used the python package scVelo⁷⁶ to run velocity analysis, pseudotime
529 analysis and a PAGA (Partition-based graph abstraction) graph⁷⁷. With that purpose we
530 exported our Seurat object to .h5dr format compatible with scVelo and followed their well-
531 documented pipeline.

532

533 Once all our clusters were assigned to one of the main stages of spermatogenesis, we
534 performed a comparison with mouse and human using one to one orthologs. We
535 downloaded the complete list of markers found by Hermann et al. for mouse and human
536 and we grouped the clusters in the three main stages of spermatogenesis and identified the
537 shared markers among flycatchers, mouse and human. The shared markers were displayed
538 using Venn plots.

539

540 **Gene expression patterns at different stages of spermatogenesis**

541 We calculated average expression of all genes per cell cluster for our merged object using
542 the AverageExpression function after using the LogNormalize function in the RNA counts
543 matrix. Then we added as metadata the location of each gene, either on the autosomes or
544 on the Z chromosomes. We subset the data to all genes expressed and to the top 500
545 expressed genes, respectively, and performed a bootstrap resampling with replacement to
546 generate confidence intervals. To test for enrichment of Z-linked genes in each cluster we
547 performed a hypergeometric test for over-representation using the R function phyper and
548 lower.tail = false.

549

550 **Molecular signatures of evolution throughout the cell clusters**

551 The d_N/d_S ratio per cluster was computed as the ratio of average d_N and average d_S for the
552 top 500 expressed genes based on collared flycatcher specific d_N and d_S values⁷⁸. We subset
553 outlier genes having a $d_S > 1$ and $d_N/d_S > 2$. Finally, we performed a bootstrap resampling to
554 generate confidence intervals.

555 We used the metadata of species in our Seurat object to perform the comparison of gene
556 expression at the different stages of spermatogenesis between the two species of
557 flycatchers. The function FindMarkers was used to perform a Wilcoxon rank sum test to
558 calculate average fold change and find DE genes between the two flycatcher species within
559 each cluster. We used the adjusted p -value with a threshold of 0.05 for significance of
560 average fold change. In order to visualize the DE genes, volcano plots were computed using
561 the package enhanced volcano. We performed a hypergeometric test on the DE genes of
562 each cluster to test for over-representation of Z-linked genes using the R function phyper.
563 To test whether there was a significant difference of DE genes between the stages, we
564 implemented a generalized linear model with binomial distribution of the response variable.
565 We used the cbind function to consider DE genes or non-DE genes as binary response
566 variable and we used spermatogenesis stage as explanatory variable. Finally, we performed
567 a GO analysis on the DE genes using the same methods described above.

568

569

570 **Author Contributions**

571 AQ and JCS conceived the study. AQ, JCS and CC collected the samples. JCS sacrificed the
572 birds. CC performed the dissections. JCS, CC and CB adapted and carried out the cell

573 suspension protocol. JCS and MS performed the single cell clustering and bioinformatics
574 analysis. JCS, MS and CFM performed and discussed the molecular evolution and statistical
575 analysis. JCS, MS, CFM and AQ discussed and interpreted all the results. JCS and AQ wrote
576 the manuscript. All authors commented and approved the final version of the manuscript.

577

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594 University.

595

596 **Ethical permits**

597 Permit for keeping flycatchers in aviaries and sacrificing maximum 17 flycatchers per year.

598 Swedish environmental protection agency Natur vårds verket (NV-01203-18) valid from

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600

601 **Competing interests:** There are no competing interests.

602 **Data availability:** All data and code will be available in a public repository (pending).

603

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785

786 **Tables**

787 Table 1. Numbers of differentially expressed (DE) genes and non-differentially expressed

788 (non-DE) genes between collared and pied flycatchers per cluster for autosome genes and Z-
789 linked genes. Mitochondrial DE genes and DE genes with unknown position in the genome
790 were excluded from this particular analysis.

Stage	Cluster	Autosomes		Z Chromosome		P-value
		DE	non DE	DE	non DE	
Somatic cells	17	0	2508	0	113	1
Somatic cells	11	0	689	0	43	1
Somatic cells	5	0	693	0	37	1
Somatic cells	16	0	2352	0	133	1
Somatic cells	12	0	1235	0	68	1
Spermatogonia	10	1	929	0	43	1
Spermatogonia	9	2	818	0	40	1
Meiosis stages	15	0	1852	0	83	1
Meiosis stages	13	0	1759	0	85	1
Meiosis stages	0	36	354	8	12	0.00044
Meiosis stages	8	5	767	0	35	1
Meiosis stages	3	39	541	4	35	0.2827
Spermatids	6	104	348	9	16	0.1092
Spermatids	1	215	397	12	23	0.6057
Spermatids	14	1	993	0	46	1
Spermatids	2	44	657	6	41	0.085
Spermatids	4	32	497	4	26	0.119
Spermatids	7	19	1160	1	50	0.5742

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796 **Table 2. DE genes related to cellular respiration and cell motility.** Subset of DE genes between collared and pied flycatchers located on the
 797 mitochondrial chromosome and on either the Z-chromosome or autosomes but coding for mitochondrial proteins.

Gene name	Ensembl Gene ID	Gene Type	Chr.	Description	DE in cluster
					3,4,1,6,0,2,8,9,10,
	ENSFALG00000016231	Mt_rRNA	MT	mt rna	5
nad2	ENSFALG00000016239	protein_coding	MT	Nadh dehydrogenase subunit 2	3,1,0,2
cox2	ENSFALG00000016248	protein_coding	MT	Cytochrome c oxidase subunit ii	3,1,6,0,2,9
atp6	ENSFALG00000016251	protein_coding	MT	Atp synthase f0 subunit 6	3,4,1,6,0,2,9
cox3	ENSFALG00000016252	protein_coding	MT	Cytochrome c oxidase subunit iii	3,4,1,6,0,2,8,9,5
cytb	ENSFALG00000016262	protein_coding	MT	Cytochrome b	3,1,6,0,2
ND6	ENSFALG00000016265	protein_coding	MT	Nadh dehydrogenase subunit 6 Sperm flagellar 2. Axoneme and	3,0
SPEF2	ENSFALG00000002091	protein_coding	Z	correct sperm head formation. ortholog: SERF1A Small EDRK-	3
	ENSFALG00000012304	protein_coding	Z	Rich Factor 1A	3,0
ACO1	ENSFALG00000001167	protein_coding	Z	aconitase 1 iron sensor Nadh:ubiquinone	3,1,2
NDUFS4	ENSFALG00000010767	protein_coding	Z	oxidoreductase subunit s4 Mitochondrial creatine kinase	4
CKMT2	ENSFALG00000012745	protein_coding	Z	(MtCK)	4,6
	ENSFALG00000016233	Mt_rRNA	MT	mt rna	1,0
nad1	ENSFALG00000016235	protein_coding	MT	Nadh dehydrogenase subunit 1	1,0
cox1	ENSFALG00000016245	protein_coding	MT	Cytochrome c oxidase subunit i	1
nad5	ENSFALG00000016261	protein_coding	MT	Nadh dehydrogenase subunit 5	1,0
UBAP2	ENSFALG00000009935	protein_coding	Z	ubiquitin associated protein Fch and mu domain containing	1
TMEM171	ENSFALG00000010257	protein_coding	Z	endocytic adaptor 2	1

TMEM174	ENSFALG00000010257	protein_coding	Z	Fch and mu domain containing endocytic adaptor 2 chaperone post translational modification	1,6	1
RPS23	ENSFALG00000005259	protein_coding	Z	ribosomal protein		1
SSBP2	ENSFALG00000012668	protein_coding	Z	single strand binding protein	1,6,0	
RPL37	ENSFALG00000002006	protein_coding	Z	ribosomal protein		6
PAX5	ENSFALG00000012983	protein_coding	Z	Paired box 5, transcription factor.		6
	ENSFALG00000010043	protein_coding	Z	POC5 centrosomal protein, involved in cell cycle		0
				Cdc28 protein kinase regulatory		
CKS2	ENSFALG00000012438	protein_coding	Z	subunit 2		0
SETD9	ENSFALG00000010986	protein_coding	Z	Set domain containing 9		2
DNAJA1	ENSFALG00000001223	protein_coding	Z	heat shock protein		2
				Mitochondrial ribosomal protein		
MRPL57	ENSFALG00000014840	protein_coding	1	I57	6,1,2,4,7	
				Mitochondrial fission regulator		
MTFR1	ENSFALG00000005475	protein_coding	2	1		1
MFN1	ENSFALG00000008386	protein_coding	9	Mitofusin 1		1

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