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1 **Letter MBE: Discoveries**

2 **DNA methylation changes in the sperm of captive-reared fish: a route to epigenetic**
3 **introgression in wild populations**

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13

Abstract

Interbreeding between hatchery-reared and wild fish, through deliberate stocking or escapes from fish farms, can result in rapid phenotypic and gene expression changes in hybrids, but the underlying mechanisms are unknown. We assessed if one generation of captive breeding was sufficient to generate inter- and/or transgenerational epigenetic modifications in Atlantic salmon. We found that the sperm of wild and captive-reared males differed in methylated regions consistent with early epigenetic signatures of domestication. Some of the epigenetic marks that differed between hatchery and wild males affected genes related to transcription, neural development, olfaction and aggression, and were maintained in the offspring beyond developmental reprogramming. Our findings suggest that rearing in captivity may trigger epigenetic modifications in the sperm of hatchery fish that could explain the rapid phenotypic and genetic changes observed among hybrid fish. Epigenetic introgression via fish sperm represents a previously unappreciated mechanism that could compromise locally adapted fish populations.

29 Captive rearing can cause rapid phenotypic and genetic changes in fish after just one
30 generation (Araki, et al. 2007; Stringwell, et al. 2014), and interbreeding between captive-
31 reared and wild fish can lead to maladaptation to natural conditions (McGinnity, et al. 2003)
32 and reduced fitness of hybrids (Araki, et al. 2007; Araki and Schmid 2010). Genome-wide
33 analyses have explained the molecular basis of phenotypic variation associated with
34 domestication in many species (Rubin, et al. 2010; Wilkinson, et al. 2013; Carneiro, et al.
35 2014) but have failed to identify common loci or strong signals of selection associated with
36 fish domestication (Ozerov, et al. 2013; Mäkinen, et al. 2015).

37 Captive-rearing in fish can result in epigenetic (methylation) changes in immune and stress-
38 related genes (Le Luyer, et al. 2017). Such epigenetic changes can respond to environmental
39 stimuli and generate phenotypic variation by modulating gene expression and function. For
40 epigenetic changes to be adaptive and evolutionary relevant, they would need to be
41 transmitted to the offspring (Bossdorf, et al. 2008; Youngson and Whitelaw 2008) and persist
42 across generations (Charlesworth, et al. 2017) to enable selection to act (Bollati and
43 Baccarelli 2010).

44 Epigenetic signatures in the sperm of zebrafish are maintained in the embryo until the mid-
45 blastula stage (Jiang, et al. 2013). If the same is true for other fish, epigenetic changes in the
46 sperm could facilitate adaptation to captivity. This would be relevant for salmonids which are
47 farmed for food or reared in hatcheries for supportive breeding programmes (Consuegra, et
48 al. 2005; Kostow 2009), and for which captive rearing causes epigenetic changes in sperm
49 (Gavery, et al. 2018). Wild salmon affected by accidental escapes from fish farms or the
50 deliberate stocking of hatchery fish often display genetic changes (Ciborowski, et al. 2007;
51 Glover, et al. 2013), altered age and size at maturation (Bolstad, et al. 2017), behavioural

52 mismatch (Houde, et al. 2010) and lower reproductive success (Theriault, et al. 2011).
53 Whether epigenetic changes also arise is not known.

54 We compared genome-wide DNA methylation profiles in the sperm of wild and hatchery-
55 reared Atlantic salmon males and their offspring to identify potentially heritable hatchery-
56 induced epigenetic modifications. Three groups of wild and hatchery-reared salmon from the
57 River Allier (France) were analysed (Tables S1-S3). Wild anadromous males (W) were
58 caught in April 2015. Hatchery H1 males were mature parr (0^+) (2014 cohort) produced from
59 re-conditioned wild males and females maintained in the hatchery for two consecutive
60 seasons, and hatchery H2 males were mature parr (0^+) (2014 cohort) from crossing females
61 hatched and reared in the hatchery with wild re-conditioned males. Both H1 and H2 were
62 reared under identical conditions. Sperm from three males of each group was used to
63 independently fertilise groups of pooled eggs of the same three wild females (Figure 1).

64 *Differentially methylated regions among parental groups*

65 The results from MethylAction and MEDIPS concurred in the identification of differentially
66 methylated regions (DMRs) and the loci affected by them. In total, 165,597 of the DMRs
67 identified among all groups coincided between MethylAction and MEDIPs. Of the loci
68 affected by those DMRs, 19,510 out of the 21,195 identified by MethylAction were also
69 identified using MEDIPS (92.05%).

70 Pairwise methylome differences using MEDIPS identified 55 significant DMRs between W
71 and H1, 22,563 between H1 and H2, and 298,980 between W and H2 (Figure 2), after
72 applying a q- value ≤ 0.05, and merging neighbouring significant windows. These DMRs
73 were overlapping or neighbouring at 47, 11,567 and 38,253 loci between W and H1, H1 and
74 H2, and W and H2, respectively (Figure S4).

75 Using MethylAction, DMRs identified from the simultaneous comparison between the three
76 parental groups were classified as 'frequent' if all the samples within each group had a
77 consistent methylation status (hyper or hypomethylated), or as 'other' if they lacked within-
78 group consistency (Bhasin, et al. 2015). Only methylation patterns of frequent and
79 statistically significant DMRs were considered further. Several of those DMRs (46,293) were
80 consistently hypermethylated in H2 compared to W and H1 individuals (Figure 1B). In total,
81 21,195 loci were affected (overlapping or neighbouring) by the +50,000 'frequent' DMRs
82 identified among all groups.

83 Of the 55 DMRs identified by MEDIPS between W and H1, 43 (78%) occurred between W
84 and H2. Of these, 35 completely overlapped with DMRs identified by Methylaction, and the
85 rest were between 2 and 1000 bp distance, all affecting the same loci. These 43 DMRs,
86 shared by both hatchery groups and different from wild individuals, appear to be distinctive
87 signatures of hatchery reared fish.

88 *Methylation comparison between parental and offspring groups*

89 Parents and offspring (mature male parr) showed significant differences in global sperm
90 methylation enrichment scores only between H2 to W males (W=1.14±0.05; H1=1.25±0.10;
91 H2=1.44±0.08; ANOVA $F_{2,6}$=7.683, p=0.0221; Tukey HSD test W-H1; p=0.42; H2-H1;
92 p=0.10; W-H2; p=0.02) (Table S1). Pairwise correlation coefficients of genome-wide
93 coverage were on average r~0.60 within groups (Figure S3).

94 The first two components of a PCA of normalized total read counts of 1000 bp sliding
95 windows explained 96.49% of the variance (Figure S5). PC1 explained 91.32%, and allowed
96 differentiation between parents and offspring ($F_{1,12}$=17.258; p=0.001), and groups within
97 generations ($F_{4,12}$=3.576; p=0.038) (Figure S5). PC1 scores differed significantly between the

98 sperm of wild parents and their offspring (post hoc Tukey HSD test; p=0.02), but not between
99 the sperm of hatchery parents and their offspring (post hoc Tukey HSD test; H1-H1off=0.98;
100 H2-H2off=0.19). This suggests that the hatchery environment (e.g. diet, confinement) had an
101 impact on the methylation status of the wild offspring sperm, born and raised under those
102 conditions. The comparisons of genetic diversity among parental groups and between parents
103 and offspring based on 927 SNPs indicated that there were no significant genetic differences
104 among groups (Fisher's exact test, P=1.00), suggesting that differences in the genetic
105 background are not responsible for the methylation differences observed.

106 For the 43 DMRs between wild and hatchery parents, all hatchery individuals (parents and
107 offspring) clustered together with the wild offspring and separately from the wild parents
108 (Figure 3 A-B) (PC1 score Kruskal-Wallis χ^2=11.99, df=5; p=0.034). Of the DMRs involved,
109 12 overlapped with genes or putative promoters, and the remaining with distal inter-genic
110 regions (Table S2a). Affected genes showing differential methylation between W and H1
111 included the transcription factor *SOX-13-lik*e (Pevny and Lovell-Badge 1997), the neuronal
112 migration protein *doublecortin-like*, expressed in fish olfactory bulb and optic tectum (Tozzini,
113 et al. 2012) and the small G protein signalling modulator 2-like, related to neural development
114 in human and mice (Yang, et al. 2007). Some of the DMRs differentiating parental groups
115 maintained the same methylation pattern in the offspring and may not have been erased during
116 early reprogramming (Table S2b). Of these, two were maintained between W and H1, 167
117 DMRs between W and H2 (overlapping genes or promoters of 73 genes), and 105 DMRs
118 between H1 and H2, affecting 24 genes (Figure S6). These results provide evidence that
119 captive rearing induces rapid epigenetic (methylation) changes in salmon sperm, some of which
120 can persist for at least one generation.

121 Variation in life history strategies (anadromous males versus mature resident males) may
122 account for some observed methylation differences between the sperm of wild and hatchery
123 males (Morán and Pérez-Figueroa 2011). However, some of these likely characterise
124 hatchery rearing, as methylation signatures among the offspring of wild fish reared under
125 hatchery conditions were more similar to those of hatchery fish than to their wild parents.
126 Furthermore, differences between parental H1 and H2 fish were stronger than those between
127 the wild and H1 groups. The regions affected include genes encoding for coiled coil-type and
128 PH domain proteins that regulate intracellular signalling networks and gene expression
129 (Kutzleb, et al. 1998) and changes to the PcG protein L3MBTL4 that regulates transcription
130 and chromatin structure, and could underlie heritable changes in gene expression (Holoch and
131 Margueron 2017). Also include the TATA-binding protein like (*tbpl1*), related to
132 spermiogenesis and embryonic development (Akhtar and Veenstra 2011), that displays
133 differential methylation between hatchery and wild coho salmon as well (Le Luyer, et al.
134 2017).

135 In the parental groups, several regions differentially methylated between the W and H2
136 parents also differed between H1 and H2, with a high degree of conservation in their
137 functions (i.e. ion transport, metabolic process, methylation; Figure S7). Even if the hatchery
138 parents (H1 and H2) had been born and raised under the same hatchery conditions, their
139 parents had spent different time in captivity (the mothers of the H2 group were born in the
140 hatchery, whereas both parents of the H1 group had a reconditioned origin, i.e. were born in
141 the wild). Thus, as the main difference between the H1 and H2 groups was the origin of their
142 mothers, the methylation signature shared between W and H1 fish, that differed from H2
143 salmon, could be the result of their maternal environment (Marshall and Uller 2007). This
144 supports a role for, potentially transgenerational, maternal effects during fish domestication
145 (Christie, et al. 2016).

146 The sperm of parents and offspring displayed distinctive methylation profiles, suggesting that
147 salmon PGCs could undergo a second reprogramming, as in mammals (Hackett and Surani
148 2013). However, some methylation marks can escape such resetting and result in epigenetic
149 transgenerational inheritance, even if only for a small number of epialleles (Daxinger and
150 Whitelaw 2012). Here, six of the common DMRs shared between W/H2 and H1/H2 were
151 maintained in the next generation, including the transcription factor EB-like, expressed
152 during embryo development (Lister, et al. 2011), the SPT20 protein, part of the SAGA
153 complex (Nagy, et al. 2009), and the corticotropin-releasing factor receptor 1-like, involved
154 in social stress and aggression (Backström, et al. 2015). This indicates a potential mechanism
155 for heritable phenotypic responses to captive rearing, although further confirmation of the
156 functional relevance of these methylation changes, including more populations, is warranted.
157 Given the important contribution that mature male parr make to the reproduction of Atlantic
158 salmon in the wild (Garcia-Vazquez, et al. 2001; Garant, et al. 2003), interbreeding of
159 hatchery-reared mature parr with wild females could potentially result in epigenetic changes
160 in wild populations.

161 Our findings suggest that at least part of the sperm epigenetic modifications associated with
162 captive-rearing remain in the offspring beyond developmental reprogramming and could
163 affect embryo fitness and performance. Hatchery-reared males could cause epigenetic
164 introgression into wild populations after just one generation if they interbred with wild
165 females, potentially disrupting local adaptation (Garcia de Leaniz, et al. 2007). The
166 importance of this mechanism in adaptation can be better advanced by further analyses of the
167 candidate genes/DMRs identified and by analysing the reversibility of these changes
168 following the cessation of hatchery rearing.

169 Gene expression changes appear associated with captive-rearing (Christie, et al. 2016), but
170 the role of epigenetics is only starting to be considered (Nätt, et al. 2012). Epigenetic
171 modifications induced by captive-rearing can influence fitness in first-generation hatchery
172 salmonids, but their inter- or transgenerational persistence has not been resolved (Le Luyer,
173 et al. 2017). Here we provide the first evidence of stability of these epigenetic modifications
174 between generations and suggest that sperm-mediated epigenetic introgression could explain
175 the rapid changes experienced by wild fish when they interbreed with hatchery-reared fish
176 (Araki, et al. 2009).

177 **Material and methods**

178 Sperm from three randomly chosen individuals from each of the male groups (W, H1 and H2)
179 was used to fertilize batches of 300 ova pooled from three wild females (100 ova/female)
180 (Supplementary methods). 125 µl of sperm from each male were pipetted onto Whatman
181 FTA Classic cards for methylation analyses. The remaining sperm was used for sperm quality
182 assessment (Caldeira, et al. 2018). Fertilised eggs from each of the parental crosses were
183 reared under identical hatchery conditions for 8 months until maturity, when sperm from 8
184 random juvenile males from each of the offspring groups was analysed for DNA methylation.

185 DNA was extracted from 6 mm pieces of each FTA card with a GenSolve kit (GenTegra
186 LLC, Pleasanton, USA), using QIAamp Blood Mini kit (QIAGEN Group) for DNA
187 purification, and the re-extracted to increase DNA recovery.

188 *Methylated DNA enrichment and analyses*

189 DNA was fragmented to <1000 bp by incubating dsDNA with NEBNext® dsDNA
190 Fragmentase® (New England BioLabs Inc.) for 30 min. Fragmented DNA was cleaned-up
191 using QIAquick spin columns (QIAGEN Group). Methylated DNA was isolated from

192 fragmented whole genomic DNA using MethylMiner™ kit from Invitrogen (CA, USA).
193 Methylated fragments were eluted using a high salinity elution buffer (2000 mM NaCl). As a
194 control, gDNA was spiked with 1 pg of synthetic methylated and non-methylated DNA
195 fragments (Methyl Miner kit, Invitrogen) before MBD-enrichment. Enriched (MBD2-
196 captured) and unbound DNA fractions were amplified using specific primers for each spike-
197 in control (Figure S1). Additional enrichment quality checks were performed (Figure S2).

198 Methylated-enriched DNA was quantified (Qubit), diluted to 0.2 ng ml^{-1} and used for library
199 preparation using Nextera-XT kit (Illumina Inc., CA, USA). Libraries were indexed for
200 multiplexed paired-end sequencing (2x125 bp read length) on an Illumina HiSeq 2500
201 platform (Illumina Inc., CA, USA).

202 After quality check using FastQC/0.11.2. and adaptor trimming (Trimmomatic/0.33, Bolger
203 et al., 2014), reads were aligned to the Atlantic salmon genome (ICSASG_v2) using Bowtie2
204 (Langmead and Salzberg 2012). MEDIPS (Lienhard, et al. 2013) was used for quality
205 control, genomic coverage estimation and to detect pairwise DMRs among and between
206 groups. We used MethylAction R (Bhasin, et al. 2015) to further assess sperm methylome
207 differences among groups. In both cases, a window size of 50 bp, and q-value cutoffs of 0.05
208 after FDR multitest correction was applied (p value (Benjamini-Hochberg) <0.05). BAM files
209 were imported to SeqMonk v1.37.1 (Andrews 2015) for visualization of mapped regions and
210 PCA. To compare the results of MethylAction and MEDIPS, adjacent 50 bp significant
211 windows were merged. BEDTools (Quinlan and Hall 2010) intersect was used to assess
212 overlapping DMRS and enable the comparison between tools. Loci affected consisted of
213 those with DMRs overlapping or neighbouring them.

214 BAM files the genome-wide MBD enrichment sequencing for a total of 9 parental male fish
215 were processed using the AddOrReplaceReadGroups utility in Picard Toolkit (Picard 2018).

216 Indel targets were identified using Target Creator in GATK 4.0.11.0 (DePristo et al. 2011)

217 and variants were exported into Golden Helix SNP & Variation Suite 8.3.3. SNPs were

218 filtered using the LD pruning utility in Golden Helix using default options (Supplementary

219 material). Genepop 4.7.0 (Rousset 2008) was used to test for global genotypic differentiation,

220 using Fisher's exact test.

221

222

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352 **Data accessibility.** Sequences have been deposited in SRA Knowledge Base under accession
353 numbers SRR8296345- SRR8296361. DMR details are included in Supplementary material
354 as well as detailed protocols. Additional sequencing results will be deposited in Dryad after
355 manuscript acceptance and are fully available from the authors for review upon request as
356 could not be directly uploaded (size >300 MB).

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362 **Author contributions.** SC & CGL designed the study; DRB carried out the sampling and
363 analyses; EV, CGL, SC & MC obtained the funding; HS carried out the SNPs analyses; all
364 authors contributed to the interpretation of the results; CGL & EV contributed to the writing
365 of the paper which was initially drafted by DRB & SC and checked and approved by all the
366 authors.

368 **Figure legends**

369 **Figure 1. Outline of the experimental design.** Parental origin of wild (W) and hatchery (H1
370 and H2) groups and their offspring. Wild adult salmon were captured from the river Allier on
371 their return to the spawning grounds, H1 salmon originated from crosses between re-
372 conditioned males and females (wild origin fish recovered and maintained in the hatchery for
373 more than 1 year after spawning) and H2 salmon originated from crosses between re-
374 conditioned males and hatchery-born females (details in supplementary material). Sperm of
375 the three groups of parents (W, H1 and H2) was used to fertilise the eggs of three wild
376 females to create the offspring. Sperm sampling points for methylation are indicated by a red
377 asterisk.

378 **Figure 2. Differentially methylated regions (DMRs). (A)** DMRs found using MEDIPS
379 showing unique and shared DMRs among groups comparisons. **(B)** DMRs found using
380 Methylaction. *Table:* Number of DMRs detected for all possible patterns of hyper- (black
381 squares) and hypomethylation (white squares). (**) Patterns with FDR <0.01; (*) Patterns
382 with FDR <0.1. 'Frequent' DMRs correspond to those where the methylation status of all the
383 samples within a group agrees (3/3). *Heatmap*: Heatmap of normalized read count
384 distributions for all 'frequent' DMRs detected. Columns represent samples, and rows DMRs.

385 **Figure 3. Clustering of parents and offspring targeting those regions that were DM**
386 **between hatchery and wild individuals in the parental group ('hatchery reared fish**
387 **distinctive signatures').** (A) PCA using normalized total read counts of 1000 bp sliding
388 windows genome wide for the target regions. (B) Clustering and Heatmap of normalized read
389 counts (log transformed) of 'hatchery reared fish distinctive signatures'. Columns represent
390 samples, and rows DMRs (the name of the closest/overlapping loci was assigned to each
391 DMR).

Figure 1.

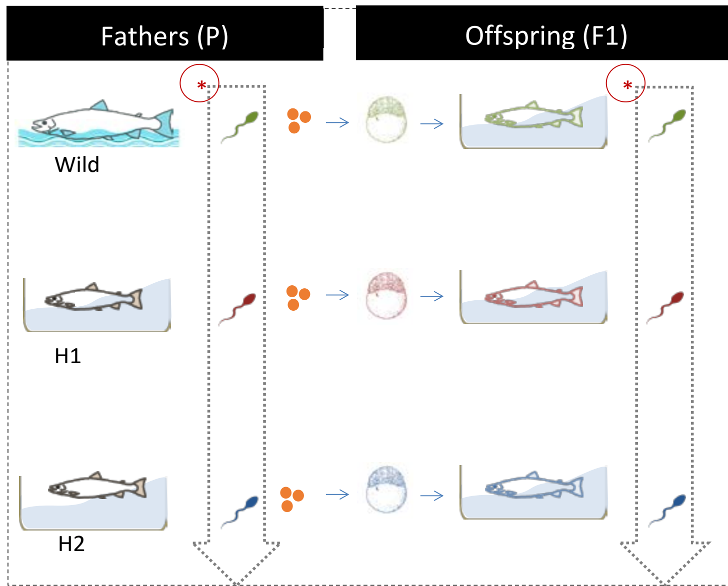
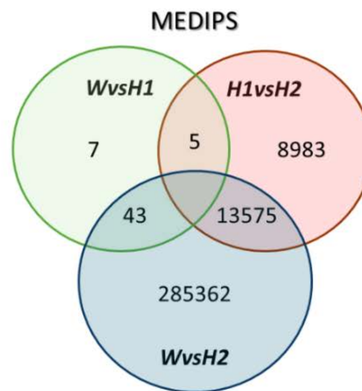


Figure 2.



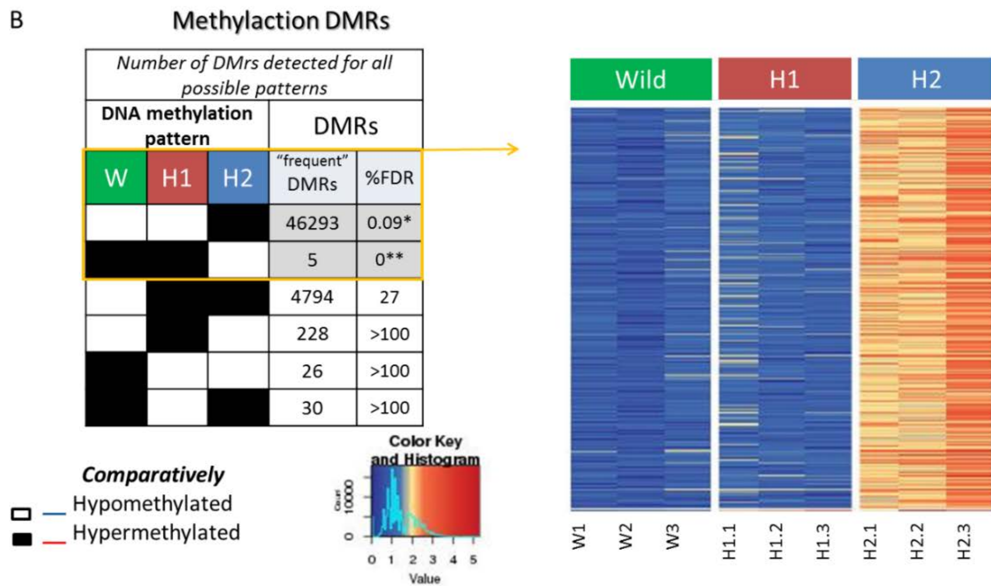
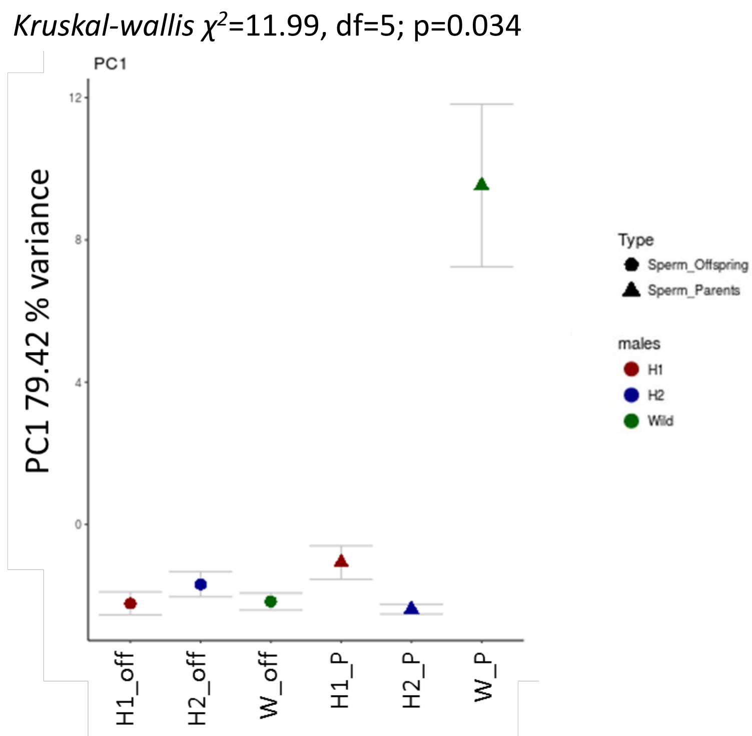


Figure 3.

A



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