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# Metabolic hormones in human breast milk are preserved by high hydrostatic pressure processing but reduced by Holder pasteurization

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1       **Metabolic hormones in human breast milk are preserved by high**  
2               **hydrostatic pressure processing but reduced by Holder**  
3                       **pasteurization**

4  
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26

### **Abstract**

27 In human milk banks (HMBs), donor milk (DM) is commonly sterilized by Holder  
28 pasteurization (HoP). High hydrostatic pressure (HHP) processing is an innovative,  
29 alternative method for DM sterilization. We evaluated the impact of HHP processing  
30 on the concentration of seven metabolic milk hormones. Eight samples of raw DM were  
31 aliquoted. One aliquot was sterilized by HoP (62°C for 30 min), and another was  
32 processed by HHP (350 MPa at 38°C). Compared with raw DM, HoP milk displayed  
33 reduced concentrations of insulin, nesfatin-1, cortisol, leptin, apelin and GLP-1, though  
34 adiponectin levels were unchanged. HHP processing maintained the levels of insulin,  
35 nesfatin-1, cortisol and leptin at their initial levels in raw DM, reduced apelin and  
36 adiponectin levels, but increased GLP-1 level. Sterilization of DM by HHP thus  
37 preserves the main metabolic hormones in human milk, underlining the interest of this  
38 method for use in HMBs.

39

40 **Key-words:** high hydrostatic pressure, holder pasteurization, human milk, hormones

## 41 1. Introduction

42 During the last decade, evidence has steadily accumulated for the importance of  
43 bioactive breast milk (BM) hormones as factors in the health of newborns and the  
44 development of infants. Experimental and clinical studies have shown that BM  
45 hormones could act directly on the newborn intestine or be absorbed and transferred  
46 into the newborn's blood circulation to reach other organs (Victoria et al., 2016; Badillo-  
47 Suárez et al., 2017; Savino & Liguori, 2008; Schueler et al., 2013). For instance,  
48 studies in rodents have demonstrated that BM leptin can be absorbed into the  
49 newborn's blood via the small intestine, and that it controls the establishment of  
50 hypothalamic neural networks involved in food intake (Casabiell et al., 1997; Bouret et  
51 al., 2004). Similarly, BM insulin was shown to reach the newborn's hypothalamus and  
52 modulate neural connections (Vogt et al., 2014), and was also implicated in gut  
53 maturation and in the reinforcement of the gut barrier (Shulman, 2002). So far, more  
54 than thirty peptidic and steroid hormones have been described in human BM (Schams,  
55 & Karg, 1986; Kratzsch et al., 2018). Their levels in BM are frequently correlated with  
56 those in maternal plasma and are modulated by maternal characteristics such as body  
57 mass index and diet (Schams, & Karg, 1986; Kratzsch et al., 2018; Andreas et al.,  
58 2014). Based on these findings, it was proposed that BM hormones may be part of a  
59 system controlling energy homeostasis and gut maturation in newborns, both directly  
60 and indirectly.

61 In preterm infants poor feeding is one of the main reasons for inadequate weight gain  
62 after birth. Immaturity of the gastrointestinal tract, feeding difficulties due to immaturity  
63 of sucking movements, low BM supply, and a lack of hepatic glycogen stores contribute  
64 to difficulty in meeting the nutritional requirements in preterm infants (McNelis et al.,  
65 2017). To ensure adequate growth of preterm babies, mother's BM is the best choice

66 for enteral feeding but, when unavailable, donor milk (DM) is often used (Boquien,  
67 2018). DM is provided by human milk banks (HMBs) which collect, screen and store  
68 DM. In order to ensure microbial safety for preterm newborns, most HMBs sterilize DM  
69 using the standard method of Holder pasteurization (HoP), commonly performed by  
70 heating DM to 62.5°C for 30 minutes (Picaud, & Buffin, 2017). Although clinical  
71 practices demonstrate that many beneficial properties of DM still persist after HoP, this  
72 pasteurization is known to partly degrade numerous heat-sensitive bioactive factors in  
73 BM such as immunoglobulins, lactoferrin, some vitamins, lysozyme, the bile salt-  
74 dependent lipase (BSSL), and some milk hormones (Picaud, & Buffin, 2017; Escuder-  
75 Vieco et al., 2018; Ley et al., 2011). As these milk compounds are important for growth,  
76 development and immunity of premature babies, methods of DM sterilization such as  
77 high-temperature short-time (HTST) or ultra-violet (UV) pasteurization, as well as  
78 sterilization by high hydrostatic pressure (HHP) processing, have recently emerged as  
79 alternatives (Wesolowska et al., 2019).

80 The HHP process applies high pressures in a temperature and time-controlled manner.  
81 This method has been used for 25 years for sterilization in food processing and was  
82 recently adapted for DM sterilization (Wesolowska et al., 2019; Demazeau et al., 2018;  
83 Wesolowska et al., 2018). Demazeau *et al.* showed that HHP processing using four  
84 cycles of a moderate pressure (350 MPa) during 5 minutes at 38°C is effective for  
85 sterilizing DM and conserves lactoferrin, BSSL enzyme and lysozyme levels close to  
86 those in raw DM (Demazeau et al., 2018). The aim of the present study was to  
87 investigate if HHP treatment also conserved several key metabolic hormones in DM  
88 including six peptidic hormones (*i.e.* leptin, insulin, adiponectin, apelin, GLP-1 and  
89 nesfatin-1), as well as one steroid hormone (cortisol). These hormones were also  
90 measured in raw DM and in DM pasteurized by HoP for comparison.

91

## 92 **2. Materials and Methods**

### 93 **2.1. Milk collection and processing**

94 Sample collection, preparation, and the experimental design of the present study  
95 are shown in Figure 1. Frozen DM samples from 11 donors were provided by the  
96 regional HMB (Lactarium Régional de Lille, Jeanne de Flandre Children's Hospital,  
97 CHU Lille). Donors provided written, informed consent for the use of their milk for this  
98 research purpose. After thawing of milk samples, 8 different batches of DM were  
99 created under sterile conditions by mixing various volumes (from 10 to 30 ml) of all BM  
100 samples, primarily in order to homogenize DM composition between batches (BM  
101 hormones levels display high level of inter-individual variation).

102 Three aliquots of DM were prepared for each batch: one fraction was stored at -  
103 80°C without any other treatment (raw milk sample); one fraction was subjected to HoP  
104 according to the standard pasteurization protocol (62.5°C for 30 min) in our regional  
105 HMB; the last fraction was subjected to HHP processing as previously described  
106 (Demazeau et al., 2018). Briefly, the set of HHP parameters was as follows: pressure  
107 = 350 MPa, temperature = 38°C, VA (application rate) = 1 MPa.s<sup>-1</sup>, number of cycles  
108 = 4 cycles, duration of each cycle = 5 min and a latency time with normal pressure  
109 between each cycle of 5 min. After HoP and HHP treatments, a microbiological  
110 analysis was performed to verify the efficacy of sterilization (Department of  
111 Microbiology, Centre de Biologie Pathologie, CHU de Lille). Sterilized samples were  
112 then stored at -80°C until analysis for milk hormonal content.

### 113 **2.2. Quantification of milk hormones**

114 The concentrations of hormones were determined in each milk fraction using  
115 whole milk. To check for potential matrix inhibition, each assay was optimized for  
116 linear-range detection of each hormone. Six abundant peptidic milk hormones and one  
117 steroid hormone were quantified using commercial ELISA kits: leptin (DLP00, R&D  
118 Systems, Abingdon, UK), insulin (HUEB0185, ELISA Genie, Dublin, Ireland),  
119 adiponectin (RD191023100, BioVendor, Brno, Czech Republic), apelin-12 (EKE-057-  
120 23, Phoenix Pharmaceuticals, Strasbourg, France), GLP-1 (HUF100805, ELISA Genie,  
121 Dublin, Ireland), nesfatin-1 (EK-003-26A, Phoenix Pharmaceuticals, Strasbourg,  
122 France) and cortisol (RE62111, IBL, Männedorf, Switzerland).

### 123 **2.3. Statistics**

124 Data are presented as mean $\pm$ SEM. Statistical analysis were performed with  
125 GraphPad Prism 7.0. software (San Diego, USA). Grubb's test was used to detect any  
126 outliers. Normality of variables was evaluated by a D'Agostino-Pearson test. If  
127 necessary, a log<sub>10</sub> transformation was applied to reach normality. Statistical  
128 differences were then tested by paired T-test or Wilcoxon test according to sample  
129 normality assessment results. A *p* value <0.05 was considered significant.

130

## 131 **3. Results**

132 The microbiological analyses of milk samples treated by HoP and HHP confirmed  
133 their efficacy as sterilization methods and the microbial safety for nutritional use of DM  
134 for preterm newborns (data not shown). Effects of HoP and HHP treatments on DM  
135 hormones levels are reported in Figure 2. Compared to raw milk, HoP treatment  
136 reduced dramatically insulin (-63%, Fig. 2A), nesfatin-1 (-41%, Fig. 2B), cortisol (-11%,  
137 Fig. 2C), leptin (-100%, Fig. 2D), apelin (-41%, Fig.2E) and GLP-1 (-83%, Fig.2F).

138 However, adiponectin levels were not different between raw and HoP-treated milk  
139 fractions (Fig. 2G). Conversely, HHP-processed fractions did not show any significant  
140 reduction in levels of insulin (Fig. 2A), nesfatin-1 (Fig. 2B), cortisol (Fig. 2C) or leptin  
141 (Fig. 2D) compared with raw DM. However, HHP-treatment reduced milk apelin (-20%,  
142 Fig.2E) and adiponectin (-50%, Fig. 2G) levels compared with raw DM, while GLP-1  
143 levels were increased (+64%, Fig.2F). Hence, a comparison between HoP and HHP  
144 treatments showed that HHP processing significantly increased concentrations of  
145 insulin, nesfatin-1, cortisol, leptin, apelin and GLP-1. Only milk adiponectin was  
146 reduced by 52% by HHP processing compared with its level in pasteurized HoP-treated  
147 milk (Fig. 2G).

148

#### 149 4. Discussion

150 Milk hormones are non-nutritive bioactive compounds that may exert short- and  
151 long-term beneficial health effects, as well as positively influence metabolic  
152 programming in both premature and full-term newborns (Victoria et al., 2016; Badillo-  
153 Suárez et al., 2017; Savino & Liguori, 2008; Schueler et al., 2013; Bouret et al., 2004;  
154 Vogt et al., 2014). In this study, we compared the effect of HoP (the reference method  
155 for DM sterilization in HMBs) with an alternative sterilization method, HHP processing,  
156 on the preservation of seven important metabolic hormones. We showed that HHP  
157 processing better preserves the concentrations of insulin, nesfatin-1, cortisol, leptin,  
158 apelin and GLP-1 in breast milk than HoP, and maintained these hormones at levels  
159 close to their initial levels in raw DM. We also observed that HHP process reduced milk  
160 adiponectin levels by 52%, whereas HoP did not affect this hormone in our study.



161 Few studies have characterized the effects of HoP on human breastmilk  
162 hormones and, so far, they have focused on a few well-described hormones including  
163 leptin, insulin and adiponectin (Escuder-Vieco et al., 2018; Ley et al., 2011; Vass et al.,  
164 2020 Wesolowska et al., 2018). Although discrepancies due to samples preparation or  
165 types of assay were reported, all these studies have shown that HoP reduced milk  
166 hormonal content. For example, breastmilk insulin levels were reported to be reduced  
167 after HoP treatment by 14% (Vass et al., 2020), 32% (Wesolowska et al., 2018) and  
168 46% (Ley et al., 2011); milk leptin by 77% (Wesolowska et al., 2018), 87% (Vass et al.,  
169 2020) and 90% (Escuder-Vieco et al., 2018); and milk adiponectin by 33% (Ley et al.,  
170 2011; Wesolowska et al., 2018). In the present study, HoP treatment decreased insulin  
171 by 63% and leptin by 100% but did not significantly affect adiponectin. In line with  
172 previous observations, our study demonstrates that both insulin and leptin are very  
173 sensitive components of human breastmilk and that their levels are drastically reduced  
174 by HoP. Discrepancies between studies (Escuder-Vieco et al., 2018; Ley et al., 2011;  
175 Wesolowska et al., 2018; Vass et al., 2020) could be related to differences in  
176 pasteurizer performance (Picaud, & Buffin, 2017). For adiponectin, in accordance with  
177 *Escuder-Vieco et al.* (Escuder-Vieco et al., 2018), we did not observe any effect of HoP  
178 on this hormone in DM whereas two studies have reported a 33% reduction of  
179 adiponectin level after HoP pasteurization (Ley et al., 2011; Wesolowska et al., 2018).  
180 We also observed a small, but significant, 11% reduction in cortisol after HoP. Using  
181 this same pasteurization method, Vass *et al.* (Vass et al., 2020) reported a 35%  
182 reduction of this hormone after HoP, suggesting that this steroid hormone is also a  
183 feature of DM sensitive to HoP. Finally, to the best of our knowledge, we report for the  
184 first time that the levels of nesfatin-1, apelin and GLP-1 are reduced in HoP-treated  
185 milk by 40%, 41% and 83%, respectively. This reinforces the idea that peptide

186 breastmilk hormones are largely degraded by HoP, probably leading to DM with  
187 reduced hormonal benefits for preterm newborns.

188 HHP processing was shown to protect numerous bioactive milk compounds from  
189 degradation (Picaud, & Buffin, 2017; Escuder-Vieco et al., 2018; Wesolowska et al.,  
190 2019; Demazeau et al., 2018; Wesolowska et al., 2018). We observed in this study  
191 that breastmilk insulin, nesfatin-1, cortisol, leptin, apelin and GLP-1 are all preserved  
192 at close to raw DM levels after HHP sterilization. Surprisingly, milk GLP-1 levels were  
193 found to be increased after HHP processing. This could result from the influence of  
194 hydrostatic pressure on the physicochemical properties of human milk. Indeed, human  
195 milk is a very complex biological fluid that could be characterized simply as an emulsion  
196 of fat globules in an aqueous liquid with cells components. Although, the precise  
197 distribution of GLP-1 in human milk remains unknown, due to its peptide structure GLP-  
198 1 is probably mainly located in the emulsion phase of human milk, which consists of  
199 milk fat droplets or fat-associated proteins suspended in the aqueous fraction of the  
200 milk. Documented effects of high pressure on milk lipids are scarce: two studies have  
201 shown that while milk fat globules are still present after HHP, an alteration in globule  
202 size was observed (Demazeau et al., 2018; Mahara et al., 2014). Hence, our  
203 hypothesis is that HHP treatment of DM causes the release of GLP-1 from fat globules  
204 to the aqueous phase. This mechanism was also proposed by Wesolowska A *et al.*  
205 (Wesolowska et al., 2018), who observed that a high-pressure application (ranging  
206 from 400 to 600 MPa) increased milk leptin levels in the emulsion phase of DM.

207 In this study, adiponectin was the only hormone whose levels were reduced after  
208 HHP processing. This finding is in accordance with the study of Wesolowska *et al.* who  
209 reported a drastic reduction of milk adiponectin following HHP process using various  
210 high pressures ranging from 200 to 600 MPa (Wesolowska et al., 2018). Adiponectin

211 is composed of multimer complexes of proteins in plasma (Kadowaki, & Yamauchi,  
212 2005). High-molecular-weight (HMW) adiponectin, which is the most biologically active  
213 form, is a large multimer of 12–18 subunits (Lihn et al., 2005)). This HMW adiponectin  
214 is present in human milk (Bronsky et al., 2006; Martin et al., 2006). As postulated above  
215 for GLP-1, we here speculate that HHP may have modified the physicochemical  
216 properties of human milk and altered the structure of the bioactive HMW adiponectin  
217 leading to its partial degradation. Further study is needed to test this hypothesis.

218 Previous studies have shown that HHP better preserved numerous compounds in  
219 DM than HoP, such as immunoglobins, lactoferrin, BSSL enzyme, lysozyme or  
220 vitamins (Wesolowska et al., 2019; Demazeau et al., 2018; Wesolowska et al., 2018).  
221 The present findings demonstrate that hormonal components of DM are also largely  
222 preserved by this non-thermal method. We believe that preservation of milk hormones  
223 in sterilized DM is especially important in the context of feeding preterm newborns with  
224 DM, as these babies have specific nutritional needs and metabolism, and hormones  
225 are involved in the maturation of numerous physiological systems in the early postnatal  
226 period (McNelis et al., 2017; Boquien, 2018; Platt, 2014). For example, experimental  
227 data have shown that leptin in breastmilk participates in the neonatal regulation of food  
228 intake and, along with insulin, in hypothalamic development; while insulin has been  
229 also shown to be involved in the maturation of digestive mucosa (Schueler et al., 2013;  
230 Casabiell et al., 1997). Both leptin and insulin were recently associated with early  
231 changes in the infant intestinal microbiome (Lemas et al., 2016; Gotteland et al., 2017).  
232 Milk glucocorticoids have been implicated in the maturation of the gastrointestinal tract,  
233 energetic metabolism and in long-term metabolic programming (Badillo-Suárez et al.,  
234 2017; Rose et al., 2013; Van Der Voorn et al., 2020). Adiponectin has been proposed  
235 to regulate the carbohydrate-lipid metabolism in newborns (Savino, & Liguori, 2008;

236 Kratzsch et al., 2018), while nesfatin-1 may regulate food and water intake (Schalla, &  
237 Stengel, 2018; Aydin et al., 2009; Aydin, 2010). Breastmilk GLP-1 was proposed to be  
238 involved in infant satiety, glucose homeostasis and reduction of gastrointestinal motility  
239 (Müller et al., 2019; Schueler et al., 2013). Finally, oral apelin administration was shown  
240 to control intestinal maturation by modulating cell proliferation in the digestive mucosa  
241 in developing rodents (Antushevich et al., 2015).

242

## 243 5. Conclusion

244 In conclusion, we have demonstrated that our HHP protocol (4 cycles of a  
245 moderate pressure (350 MPa) during 5 minutes performed at 38°C) preserves the main  
246 metabolic hormones in human milk, reinforcing the potential of this new sterilization  
247 method to be used to treat DM in HMBs. Through the preservation of numerous  
248 important milk bioactive factors, HHP-treatment of DM could improve growth,  
249 neurodevelopment, intestinal maturation and microbiota establishment in preterm  
250 infants who receive it.

251

252

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391 The authors declare no conflict of interest.

392

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397 **Availability of data and material:**

398 Data of this study are available upon request to authors.

399

400 **Authors contribution:**

401 LM, LT and JL drafted the manuscript. LM, LT, EM, MDL and JL did the experiments  
402 and collected the data. DL, VP, DE and FG reviewed the paper. All authors approved  
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### Figures legends

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421 **Fig. 1.** Descriptive diagram of the methodology used of this study

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423 **Fig. 2.** Concentrations of hormones in raw DM and in DM after Holder pasteurization  
424 (HoP) and High hydrostatic pressure (HHP) processing. Data are presented as  
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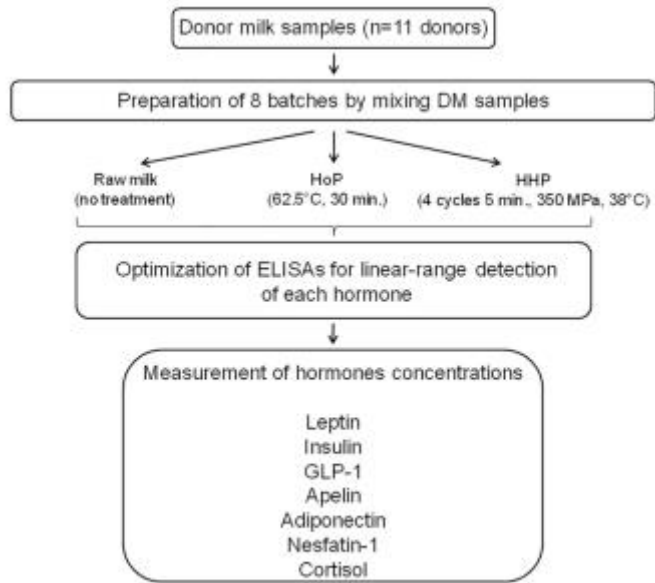


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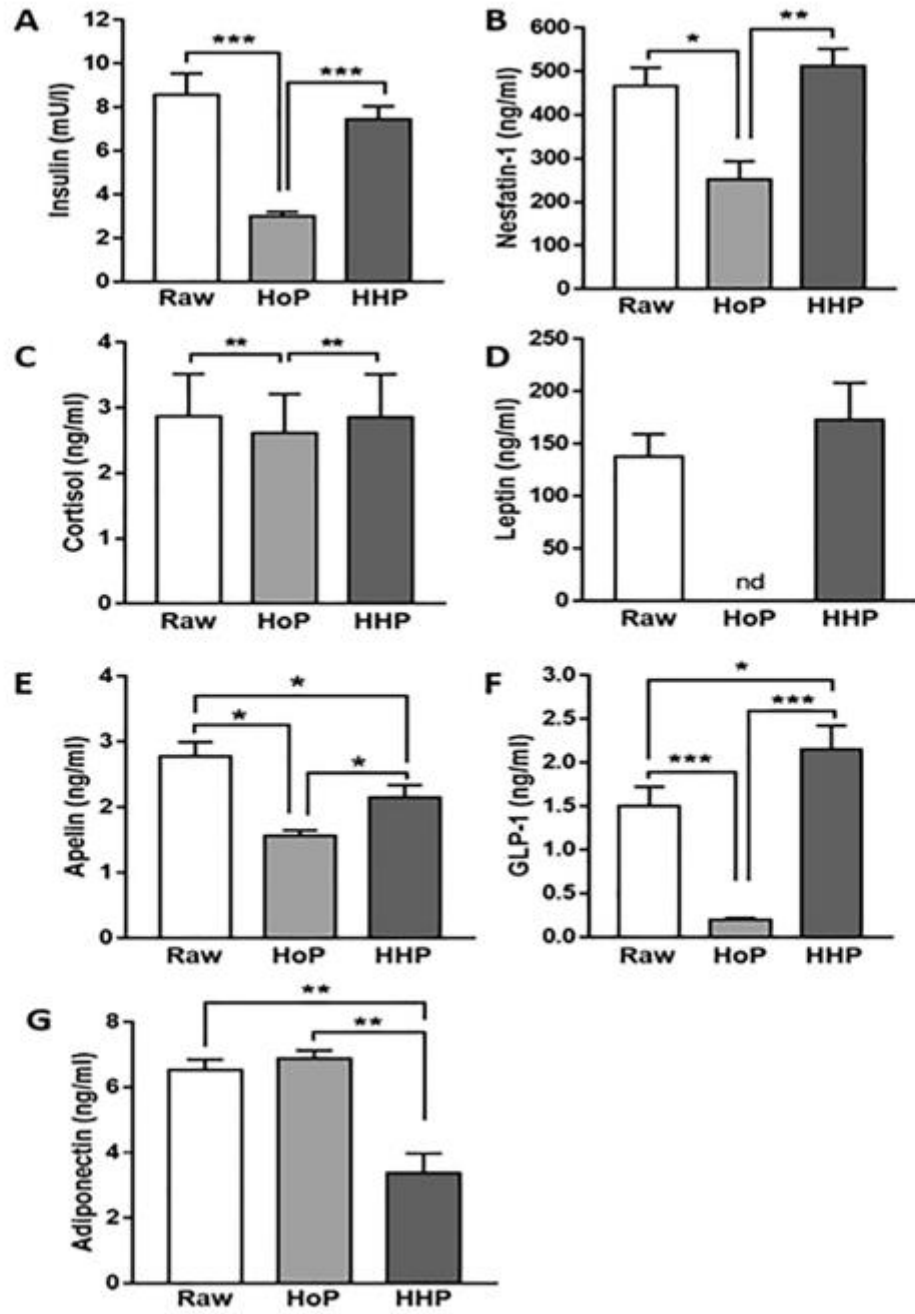


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