Early maternal separation is not associated with changes in telomere length in domestic kittens (*Felis catus*)

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Objective Studies of multiple species have found that adverse early life experiences, including childhood trauma and maternal separation, can result in accelerated telomere shortening. The objective of this study was to determine if premature separation from the mother affected telomere length in domestic kittens (*Felis catus*). Subjects were 42 orphaned kittens and 10 mother-reared kittens from local animal rescue groups and shelters. DNA was extracted from whole blood collected from kittens at approximately 1 week and 2 months of age. Telomere length was assessed by qPCR (quantitative polymerase chain reaction) from a total of 86 samples and expressed as a ratio of telomere PCR relative to a single copy gene PCR (T/S).

Results A generalized linear mixed model found there were no detectable differences in telomere length based on survival ($F_{1, 76.2} = 3.35$, $p = 0.07$), orphan status ($F_{1, 56.5} = 0.44$, $p = 0.51$), time point ($F_{1, 43.5} = 0.19$, $p = 0.67$), or the interaction between orphan status and time ($F_{1, 43.5} = 0.86$, $p = 0.36$). Although in other species, telomere shortening is commonly associated with aging, even early in life, we did not find evidence for telomere shortening by two months of age. Our results suggest that the experience of early maternal separation in domestic cats who are subsequently hand-reared by humans does not accelerate telomere shortening compared to mother-reared kittens, at least in the first few months of life.

1 Early maternal separation is not associated with changes in telomere length in domestic kittens

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18   **Abstract**

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27   ratio of telomere PCR relative to a single copy gene PCR (T/S).

28   **Results**

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31   ($F_{1, 43.5} = 0.19$, $p = 0.67$), or the interaction between orphan status and time ($F_{1, 43.5} = 0.86$, $p =$

32   0.36). Although in other species, telomere shortening is commonly associated with aging, even

33   early in life, we did not find evidence for telomere shortening by two months of age. Our results

34   suggest that the experience of early maternal separation in domestic cats who are subsequently

35   hand-reared by humans does not accelerate telomere shortening compared to mother-reared

36   kittens, at least in the first few months of life.

37

38   **Keywords:** telomeres, biological aging, domestic cats, maternal separation, telomere attrition

Introduction

Early life experiences can impact mental and physical health across the lifespan (Felitti et al. 1998). In particular, adverse childhood experiences (ACEs) have received significant attention in the medical and psychological literature because of the potential harm they can cause, including increased risk for future alcohol and drug abuse, depression, and poor health outcomes (Felitti et al. 1998). In humans and rodents, inadequate maternal care leads to increased fearfulness and negative affect (Hane & Fox 2006), stereotypies (Latham & Mason 2008), increased fear and panic in adults (Caldji et al. 1998; Nishi et al. 2014), and decreased cognitive abilities, including spatial navigation (Liu et al. 2000) and memory (Zalosnik et al. 2014).

Studies in multiple species have found that adverse early life experiences and other stressors can also result in accelerated telomere shortening (Chatelain et al. 2020; Cram et al. 2017; Gil et al. 2019; Nettle et al. 2017; Price et al. 2013; Xavier et al. 2018). Telomeres are highly conserved, non-coding regions of repetitive nucleotide sequences at each end of a chromosome that protect the genome during cell replication by preventing genes from truncating. Cells have a limited reproductive capacity, referred to as the "Hayflick Limit" (Hayflick & Moorhead 1961). Telomeres shorten during mitosis and once they reach a threshold the cell stops reproducing and enters a state of senescence or dies (Allsopp et al. 1995). Because of this process, telomeres also are associated with aging and the remaining capacity of a cell to replicate (Aubert & Lansdorp 2008; Chiu & Harley 1997). Telomeres are subject to more shortening early in life (Frenck et al. 1998), and telomere length is a strong predictor of lifespan and mortality in multiple species (Bize et al. 2009; Cram et al. 2017; Heidinger et al. 2012). Thus, adverse experiences early in life may have more influence on telomere length and long-term health and life-history than do adverse experiences later in life (Ridout et al. 2017).

62   Previous studies have demonstrated the effects of adverse early life experiences on telomere

63   length. Among European starlings, an early life competitive disadvantage (being smaller than

64   other brood members) led to more rapid telomere attrition by 12 days of age compared to birds

65   who were larger than the rest of their brood (Nettle et al. 2015). Meerkat pups born into larger

66   groups of young have shorter telomeres due to increased competition for access to milk (Cram et

67   al. 2017). Maternal stress also can shorten telomeres. For example, an increased rate of telomere

68   loss was found in female offspring of laying zebra finches treated with corticosterone compared

69   to controls and the offspring of zebra finches treated with 17-β-estradiol (Tissier et al. 2014).

70   Social isolation shortens telomeres (Aydinonat et al. 2014), but there have been few studies

71   of the effects of maternal presence or care on telomere length. Children in rural areas of China

72   who were cared for by extended family had shorter telomeres compared to children raised with

73   their mothers (Chen et al. 2019), and individuals who described their parents as neglectful had

74   shorter telomeres than those who did not (Enokido et al. 2014). Similar effects are found in non-

75   humans; Welsh pony foals that were abruptly weaned and separated from their mothers showed

76   higher levels of stress and shorter telomeres compared to foals who were progressively weaned

77   (Lansade et al. 2018). Rhesus macaques reared by their mothers had longer telomeres than

78   monkeys who were raised with peers or in isolation (Schneper et al. 2016).

79   Kittens have frequent interactions and physical contact with their mother during the nursing

80   and weaning periods, which typically last until at least two months of age (Albonetti 1988).

81   Some kittens become inadvertently separated from their mothers before weaning and are

82   subsequently hand-reared by humans. Hand-raising of kittens has become increasingly common

83   as animal shelters improve their ability to care for orphaned neonatal kittens. Orphaned neonatal

84   kittens have a high rate of mortality (Little 2013), are at risk for abnormal behaviors (Delgado et

85   al. 2020), and show increased distress vocalizations and activity during a nest separation (Lowell

86   et al. 2020), compared to mother-reared kittens. To better understand the effects of early

87   maternal separation on domestic cats, the purpose of the described study was to investigate

88   whether premature separation from the mother affected telomere length in kittens at two time

89   points.

90   Previous studies in adult cats have established a relationship between telomere length and

91   aging (McKevitt et al. 2003), and disease processes such as kidney failure (Quimby et al. 2013).

92   These and other studies have established the feasibility of measuring telomere length in cats

93   (Brümmendorf et al. 2002; Pang & Argyle 2009). To our knowledge, this is the first study

94   investigating the effect of early maternal separation on telomere length and telomere dynamics in

95   kittens.

96   If maternal separation has deleterious effects, orphaned kittens could show shorter telomeres

97   compared to control kittens. We expected similar telomere length in both groups in the first week

98   of life, as all kittens who were separated from their mothers would have experienced the

99   separation recently. We predicted that all kittens would experience some telomere shortening by

100   eight weeks of age due to the normal process of aging, and we specifically hypothesized an

101   interaction effect, such that kittens who were separated from their mothers early in life (orphans)

102   would have significantly shorter telomeres than kittens raised by mothers (mother-reared) at

103   eight weeks of age.

104   **Methods**

105   All animal procedures were approved by the Animal Care and Use Committee at the

106   University of California, Davis, under Protocol #20379.

107   *Study Animals*

108   Subjects were 42 orphaned kittens (OR) and 10 mother-reared (MR) kittens from local rescue

109   groups and shelters. Orphans had been turned into shelters or rescue groups without their

110   mothers by members of the public. All kittens were entered into the study before they were 7

111   days of age. Age was assessed by an experienced veterinary technician or shelter rescue staff, as

112   determined by known date of birth, or as estimated by presence of umbilical cord, weight, ear

113   position, and degree of eyes opening (Little 2011). Orphaned kittens were raised in foster homes

114   and were housed in incubators set to 80-90° F, and with relative humidity set to 50-60% (Little

115   2013; Peterson 2011). Incubators included a heating pad and bedding. All orphans were fed

116   commercial kitten formula (Breeder's Edge®, Revival Animal Health, Orange City, Iowa) until

117   weaned, when they were fed a commercial dry and wet food (Purina Pro-Plan Kitten Formula®,

118   St. Louis, MO). Mother-reared kittens were also cared for in foster homes; they consumed their

119   mother's milk until weaned onto the same type of wet and dry kitten food as the orphans

120   received. Foster caretakers gave written consent for fostered kittens to participate in the study.

121   Nine OR kittens died while in foster (average age of death: 20 days, SD: 11.9 days, range 6 to 42

122   days); the remaining kittens were adopted into homes after being weaned and neutered.

123   *Experimental Procedures*

124   Blood (200 µl) was drawn from each kitten via jugular venipuncture by a veterinarian or

125   veterinary technician when kittens were approximately 1 week and two months of age (mean age

126   of first blood sample: OR: 8 days, SD: 2 days, MR: 10 days, SD: 1.2 days, range 6-13 days;

127   mean age of second blood sample: OR: 69 days, SD: 9.3 days, MR: 59 days, SD: 4.3 days, range

128   50-93 days). Blood samples were placed into EDTA tubes then transferred to a cryotube and

129   stored at -80° C until analysis.

130   A total of 96 blood samples were collected, and 86 samples were used in the final analysis.

131   Blood from 28 OR and six MR kittens was collected and analyzed at both time points. Only one

132   sample (from Time 1) was collected and analyzed from each of the nine orphans who died. There

133   were an additional five OR and four MR kittens from whom blood was collected and analyzed

134   only for Time 2. Three samples (all orphans) did not produce sufficient DNA, and four samples

135   were used during assay development and were not included in the final dataset. Due to

136   experimenter error, three of the blood samples from MR kittens were misplaced, which left 86

137   viable samples for analysis (Table 1).

138   *Quantification of telomere length*

139   The telomere length measurement assay was adapted from Cawthon (Cawthon 2002; Park et

140   al. 2013). Telomere lengths were assessed by qPCR (quantitative polymerase chain reaction)

141   and expressed as a ratio of telomere PCR relative to a single copy gene PCR (T/S). The qPCR

142   analysis was done on a Roche 480 LightCycler qPCR machine, using the 'Absolute

143   quantification/2nd derivative max' automated algorithm, which calculates the point at which the

144   maximum change in slope of the fluorescence amplification curve becomes the crossing point.

145   The 'T' qPCR reaction and 'S' qPCR reactions were carried out sequentially.

146   The measurements were made from DNA extracted from whole blood. DNA extract samples

147   were stored at -80˚C. The DNA extraction includes an RNAse step and was carried out in a

148   biosafety cabinet. The quality of the DNA was measured spectroscopically, using a

149   Nanodrop2000. Genome integrity for these samples was verified by separating 100ng of each

150   DNA extract on a .8% agarose gel and no degradation was observed. All samples used for

151   telomere measurement met the criteria, 260/280 nm ratios, $1.7 < n < 2.0$, 260/230 nm ratios > 1.0,

152   and were considered free of contaminants.

153   Whole blood was treated with a 'cell lysis' solution (Qiagen, QIAmp DNA blood mini kit,

154   #51106) and the lysate was spun through a column that retained the DNA. Subsequent washing

155   and elution released the purified DNA from the column. The DNA extracted from blood from

156   each animal was diluted to 10ng/ul in a 96-well stock plate. A liquid handler was used to pipet

157   triplicate reactions from the 96-well DNA plate to a 384-well 'T' (telomere) reaction plate, each

158   well containing 7.5ul of PCR reaction mixture to give 2.5ng/ul final DNA concentration.

159   Immediately after the T reactions were put into the PCR machine, the same DNA stocks were

160   used to pipet triplicate reactions on a separate 384-well 'S' (single copy gene) reaction plate.

161   For the feline single copy gene (S), primers were made to amplify feline GAPDH. For the

162   forward primer, we used the sequence (5'->3') GTGGTGAAGCAGGCATCAGA and for the

163   reverse primer, we used CACTGTTAAAGTCGCAGGAGACA. Both primers were used at a

164   concentration of 1µM in the PCR reaction. The primers used for the telomere (T) PCR were Tel

165   C [5'-TGTTAGGTATCCCTATCCCTATC-3'], at a final concentration of 200 nM, and Tel G

166   [5'-ACACTAAGGTTTGGGTTTGGGTT-3'], at a final concentration of 400 nM.

167   Both telomere and single copy gene PCR reactions were performed with QuantiFast SYBR

168   Green PCR Kit (QIAGEN) on a Roche LightCycler 480 Realtime PCR machine (LC480; Roche

169   Diagnostics Corporation, Indianapolis, IN). T and S reactions were 10ul with stock 2x Quantifast

170   mix (5ul/reaction). The Quantifast mix contains a proprietary fluorescent polymerase for PCR.

171   The concentration of DNA in all experimental reactions was 2.5ng/ul.

172   The thermal PCR cycling profiles were as follows: for T (telomeric), denature at 95°C for 15

173   minutes, one cycle; denature at 94°C for 15 seconds, anneal/extend at 49°C for 15 seconds, one

174   cycle. Denature at 94˚ for 15 seconds, anneal/extend at 49˚C for 30 seconds, one cycle. Melt at

175   95C˚ for 15 seconds, anneal at 62˚C for 10 seconds, 74˚C for 15 seconds with fluorescence data

176   collection 32 cycles. For S (single copy gene), denature at 95°C for 10 minutes, one cycle;

177   denature at 94°C for 15 seconds, anneal at 60°C for 60 seconds with fluorescence data collection,

178   45 cycles. A standard curve was created from two-fold serial dilutions of pooled genomic DNA

179   from four OR kittens, from which the concentrations of telomere reactions and single copy gene

180   reactions were determined. A 5-point, 2x-dilution standard curve was made of pooled genomic

181   DNA from four OR kittens, starting with 10ng/ul, and ending with .312ng/ul. At least three runs

182   (T and S paired runs) were carried out for each sample. The efficiency of every T run was greater

183   than 90% and the efficiency of every S run was greater than 95%. The R^2 For T1, S1, T2, and S2

184   runs were 1.0, .99, .99, and 1.0 respectively.

185   Genomic DNAs from the same 4 kittens were plated individually as positive controls, to

186   serve as quality controls for each run. Three pairs of T and S PCR reactions were run on different

187   days and the T/S PCR ratio was calculated for each pair. If, after three runs, a value looked like

188   an outlier, it was discarded and an additional run was made. For this plate of samples, the inter-

189   plate CV (the standard deviation/average of the 2 T/S values) for every sample was averaged

190   and came to 3%.

191   *Statistical analyses*

192   All data were analyzed using SAS University Edition (SAS Institute Inc, Cary, NC). To

193   assess the effect of orphan status and age on telomere length, we used a linear mixed model

194   (LMM) in PROC MIXED including survival (Y/N), orphan status (Y/N), time point (1 or 2), and

195   the interaction between orphan status and time point as predictors, with telomere ratio as the

196   dependent variable. The residuals of the LMM met the assumptions of normality (q-q plot) and

197   homogeneity (Levene's test, $p > 0.05$), and data from all groups also met the assumption of

198   equality of variance (all Satterthwaite tests, $p > 0.05$). Litter and kitten ID (nested within litter)

199   were included as random effects to account for repeated measures of the same individuals and

200   the effects of relatedness on kittens from the same litter. Telomere lengths followed a normal

201   distribution (Kolmogorov–Smirnov test = 0.07, $p > 0.15$) and we used a Pearson's correlation to

202   assess the relationship between telomere lengths at time points 1 and 2 for kittens who had been

203   sampled twice. We also performed a post-hoc sensitivity analysis in G*Power 3.1.9.2 (University

204   of Kiel, Germany; Faul et al. 2007) to determine the effect size that would have been necessary

205   to find a difference between groups. Figures were created in R 4.0.2 (R Foundation for Statistical

206   Computing, Vienna, Austria).

207   **Results**

208   ˙Telomere ratios ranged from 0.63 to 1.44 ($x = 0.95$, $SD = 0.14$). The model found no effect of

209   survival ($F_{1,\,76.2} = 3.35$, $p = 0.07$), orphan status ($F_{1,\,56.5} = 0.44$, $p = 0.51$), time point ($F_{1,\,43.5} =$

210   0.19, $p = 0.67$), or the interaction between orphan status and time point ($F_{1,\,43.5} = 0.86$, $p = 0.36$);

211   Figure 1). The results were the same when assessing only the 34 kittens from whom we had two

212   blood samples (orphan status ($F_{1,\,45.4} = 0.75$, $p = 0.39$), time point ($F_{1,\,32} = 0.01$, $p = 0.91$), or the

213   interaction between orphan status and time point ($F_{1,\,32} = 0.72$, $p = 0.40$)).

214   There was a small but statistically significant correlation between telomere length at time 1

215   and time 2, r (34) = 0.36, $p = 0.04$; Figure 2. When comparing by orphan status, the correlation

216   for MR kittens was r (6) = 0.80 ($p = 0.06$), and for OR kittens was r (28) = 0.28 ($p = 0.14$),

217   neither of which was statistically significant.

218   *Sensitivity analysis*

219   Because of the null findings between groups, the post-hoc assessment of the sensitivity of the

220   analysis allowed us to determine the effect size that would have been necessary to find a

221   difference between groups. With adequate power ($\beta = 0.80$, $\alpha = 0.05$), to find a statistically

222 significant difference between the number of OR and MR kittens we examined at each time point

223 would have required a large effect size (Cohen's $d = 1.11$ and 0.88 respectively). This would

224 have equaled a difference in T/S ratio between groups of at least 0.13 for Time 1 and 0.14 for

225 Time 2. For the pooled data for all kittens, to find a difference between the two time points

226 would have required a medium effect size of Cohen's $d = 0.54$, which would have equaled a

227 difference between groups in T/S ratio of at least 0.08. The means of both groups were very

228 close (0.98 and .97 for Time 1; 0.98 and 0.93 in Time 2), and the differences resulted in effects

229 sizes of 0.07 (Time 1) and 0.35 (Time 2). If our findings came from representative samples, we

230 would have needed samples from more than 5000 and 200 kittens, respectively, to identify a

231 statistically significant effect at either time point.

232 **Discussion**

233   We found no detectable differences in telomere length between orphaned and mother-reared

234 kittens. We predicted that by approximately two months, orphaned kittens would have shorter

235 telomeres than mother-reared kittens and would experience more telomere attrition. Although

236 telomere shortening is commonly associated with aging, we also did not find evidence for

237 telomere shortening in either group of kittens between the first week of life and approximately

238 two months later.

239   Some previous studies have found deleterious effects of early maternal separation, including

240 telomere shortening, in other species (Chen et al. 2019; Enokido et al. 2014; Lansade et al. 2018;

241 Schneper et al. 2016). Previous studies have found that being orphaned increases behavioral

242 signs of stress and other abnormal behaviors compared to mother-reared kittens (Delgado et al.

243 2020; Lowell et al. 2020). However, for domestic cats, being raised by humans in the absence of

244 the mother may also have some protective effects. Kittens are altricial and unlikely to survive

245   when orphaned without outside care. Being raised by humans may be more like progressive

246   weaning; foals who were gradually weaned experienced less telomere shortening than abruptly

247   weaned foals (Lansade et al. 2018). All of the orphans in our study were maintained at consistent

248   temperatures and humidity and were bottle-fed, which may have lowered stress and prevented

249   competition for food as is experienced in many young animals (Hudson & Trillmich 2008),

250   possibly preventing telomere loss. Further research is needed to better understand whether

251   human care can compensate for the impact of maternal loss in kittens.

252   Alternatively, it is possible that the mothers in our study were under an increased stress level,

253   thereby shortening the telomeres of the mother-reared kittens. This explanation seems unlikely as

254   all mother cats were social with humans, and housed in quiet, private, indoor environments.

255   Instead, it appears that any potential stress in cats related to being orphaned does not have a large

256   impact on telomere length. Cortisol reduces the production of telomerase (Choi et al. 2008), an

257   enzyme which may preserve telomere length (Boccardi & Paolisso 2014). Studies of stress in

258   adult cats have not consistently found a strong correlation between cortisol and other signs of

259   stress (Fukimoto et al. 2020; Gourkow et al. 2014), suggesting one potential explanation for why

260   orphaned kittens may show more behavioral signs of stress but no telomere shortening compared

261   to mother-reared kittens. Further studies are needed to explore whether stress increases cortisol

262   level in neonatal kittens.

263   A longitudinal approach is useful for assessing the effects of aging on telomere length. We

264   did not track kittens into adulthood; ideally, measurements of survival and telomere length would

265   be taken from cats at multiple time points to assess whether premature maternal separation has

266   any long-term effects on health. Among kittens who survived to two months of age (100% of

267   mothered kittens and 79% of all orphans), we found no evidence of an impact of being orphaned

268   on telomere length. It is possible that our inability to find signs of telomere shortening in either

269   study group may have been due to the short time interval between sample collection

270   (approximately 8 weeks). Longitudinal studies of young birds, (Nettle et al. 2017; Nettle et al.

271   2015) sheep (Fairlie et al. 2016), adult laboratory mice (Cai et al. 2015), and Sudanian grass rats

272   (Grosbellet et al. 2015) found significant shortening of telomeres within similar or smaller time

273   frames (Bateson 2016). However, we cannot know if rates of attrition across species are

274   comparable.

275   Rather than finding telomere shortening, we found a correlation between relative telomere

276   length at each time point. Kittens with relatively shorter telomeres at time 1 also had relatively

277   shorter telomeres at time 2. Some orphans appeared to have increased relative telomere length,

278   whereas a decreased relative telomere length was found in others, suggesting more variability

279   among orphans.

280   Our study had some limitations. We had difficulty procuring sufficient blood samples from

281   mother-reared kittens, leaving us with imbalanced groups and power sufficient only to detect

282   medium to large effect sizes. Our sample may also be biased by the fact that we could not re-test

283   nine kittens (all orphans) who died after their initial blood sample was collected. Our model

284   suggested that the initial telomere lengths of kittens who died were not different from the rest of

285   the population sampled, and we did not find any differences between surviving orphans and

286   mother-reared kittens. Finally, there are different ways to assess telomere length, including

287   qPCR, Q-FISH, TRF analysis, and TeSLA (Lai et al. 2018; Montpetit et al. 2014). qPCR offers

288   the benefits of being easy to perform and requiring small amounts of DNA. However, qPCR can

289   only provide relative, rather than absolute telomere length, and is less sensitive to short telomeres

290   or telomeropathies (Gutierrez-Rodrigues et al. 2014; Lai et al. 2018). Thus, using qPCR may

291   have reduced our ability to find differences between our two populations of interest.

292   **Conclusions**

293   Although there may be some reported deleterious effects of early maternal separation in

294   domestic cats (Ahola et al. 2017; Delgado et al. 2020; Lowell et al. 2020), we did not find that

295   orphaned kittens had shorter telomeres than non-orphans at approximately one week or two

296   months of age. If differences do exist between the two groups, they are likely small and of

297   unknown clinical significance. Further research, ideally with larger samples, should assess

298   whether there are differences between orphaned and mother-reared populations later in life.

299

300   **Abbreviations**

301   **MR:** mother-reared

302   **OR:** orphaned

303   **PCR:** polymerase chain reaction

304   **qPCR:** quantitative polymerase chain reaction

305   **S:** single copy gene

306   **T:** telomere

307

308   **Authors' contributions**

309   MD, CATB, MB and KV designed the study. MD and KV were responsible for data collection.

310   DS conducted PCR/telomere analyses. MD analyzed the data and was a major contributor in

311   writing the manuscript. All authors read and approved the final manuscript.

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481

482 **Figure Legends**

483   Figure 1. Boxplots of telomere lengths with sample sizes for mother-reared and orphaned

484 kittens at 1 and 8 weeks of age summarized from raw data. Boxplots include median value, IQR

485 (interquartile range), and outliers.

486

487   Figure 2. Pearson's correlations between telomere lengths at approximately one week and

488 two months of age for six mother-reared kittens ($r = 0.80$, $p = 0.06$) and 28 orphaned kittens ($r =$

489 0.28, $p = 0.14$) for whom repeated samples were collected. The overall correlation for all

490 repeated samples was $r(34) = 0.36$, $p = 0.04$.

491

Figure 1

Boxplots of telomere lengths with sample sizes for mother-reared and orphaned kittens at 1 and 8 weeks of age summarized from raw data.

Boxplots of telomere lengths with means and sample sizes for mother-reared and orphaned kittens at 1 and 8 weeks of age summarized from raw data. Boxplots include median value, IQR (interquartile range), and outliers.

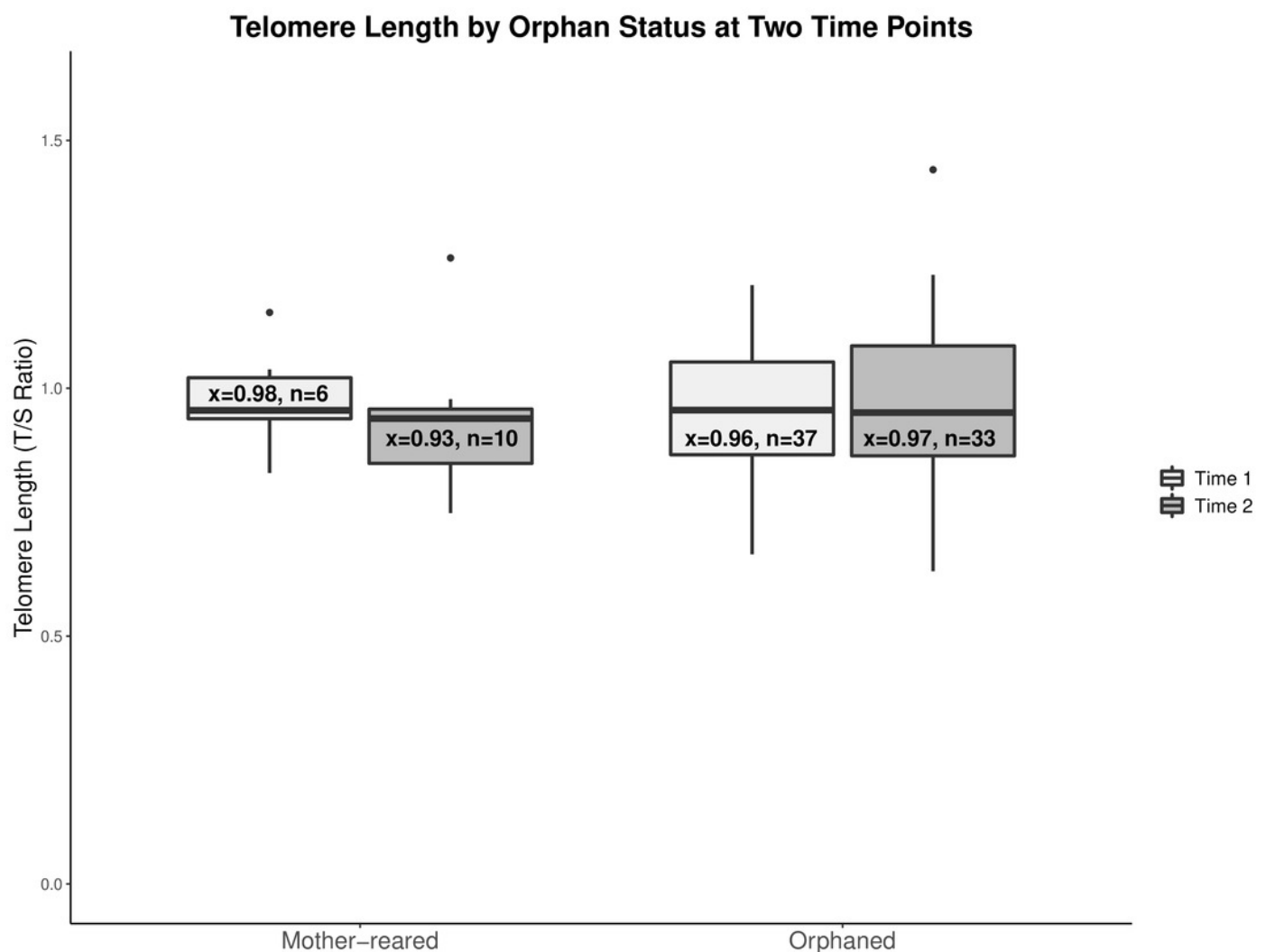
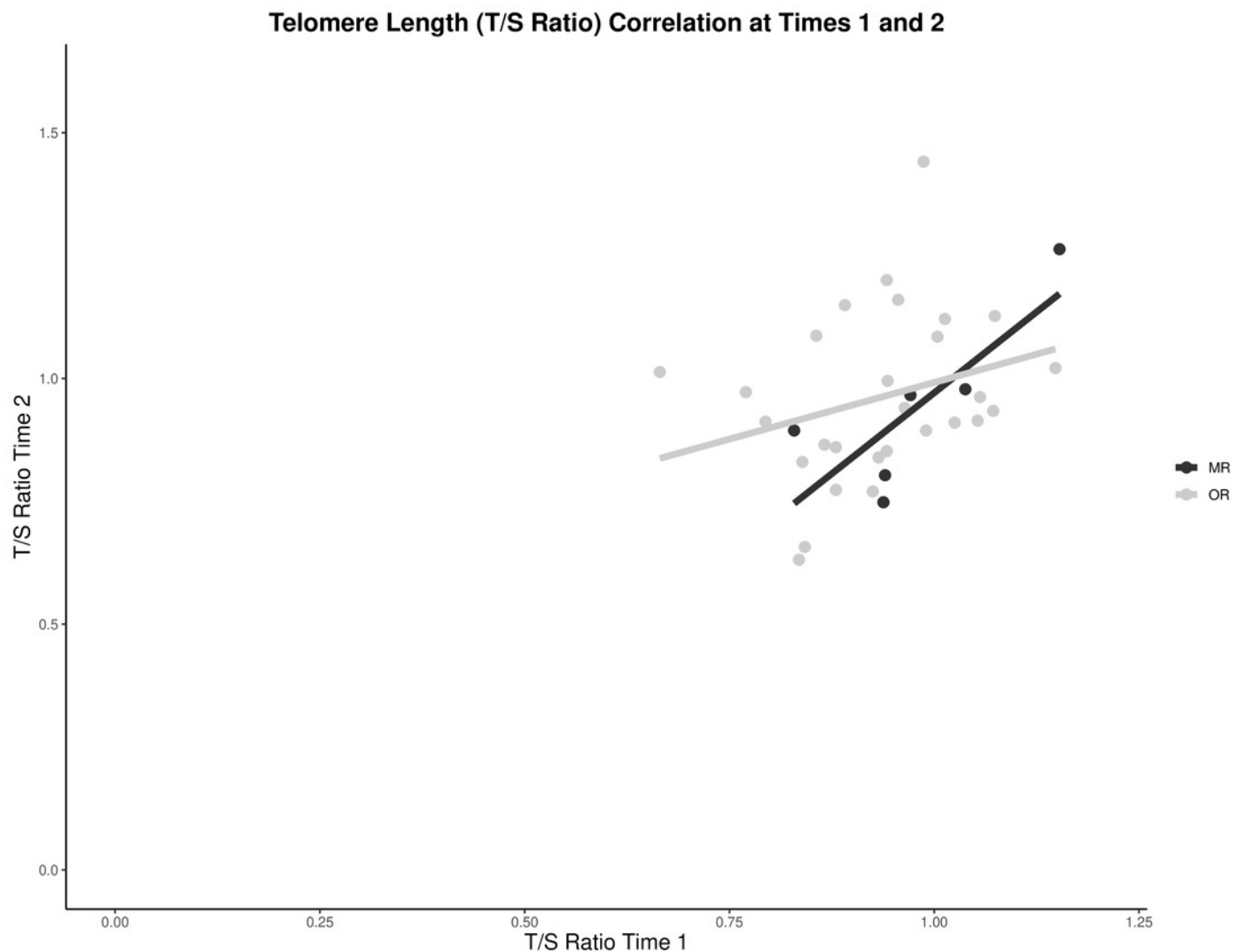



Figure 2

Scatter plot of correlations between telomere lengths at two time points.

Pearson's correlations between telomere lengths at approximately one week and two months of age for six mother-reared kittens (r = 0.80, p = 0.06) and 28 orphaned kittens (r = 450 0.28, p = 0.14) for whom repeated samples were collected. The overall correlation for all repeated samples was r(34) = 0.36, p = 0.04.

Number of blood samples acquired and used in analyses.

Number of blood samples acquired and used in analyses from mother-reared and orphaned kittens at Time 1 (one week) and Time 2 (two months). The number of repeated samples from each group is indicated in lighter text and parentheses.

1   Table 1. Number of blood samples acquired and used in analyses from mother-reared and orphaned
2   kittens at Time 1 (one week) and Time 2 (two months). The number of repeated samples from each group
3   is indicated in lighter text and parentheses.

Source	Samples not used in final analyses	Analyzed samples Time 1	Analyzed samples Time 2	Total samples collected
Mother-reared kittens	3 lost due to experimenter error	6[a]	10[b]	19
Repeated samples		(6)	(6)	
Orphaned kittens	4 assay development 3 insufficient DNA	37[c]	33[d]	77
Repeated samples		(28)	(28)	
Total	10	43	43	96

4   Note: all ten samples not used in final analyses were acquired from Time 1.
5   [a]samples from 3 litters
6   [b]4 litters
7   [c]16 litters
8   [d]17 litters