Stressful times for women - Increased physiological stress in Neolithic females detected in tooth cementum

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1. Introduction

The first demographic transition in human (pre)history happened in the Neolithic period when the global population size dramatically increased (Bocquet-Appel, 2011). This is a period of change from the hunter-gatherer-fisher lifestyle to food production, cultivation of animals and animal breeding that occurred in the early Holocene and presents the transition from the Mesolithic to the Neolithic period in Europe. It has been hypothesized that the Neolithic population boom was caused by the increase in female fertility due to sedentary lifestyle and availability of high calorie food (Bocquet-Appel, 2008).

Support for this hypothesis comes from an observed increase in the proportion of subadult individuals in the first prehistoric farming skeletal populations all over the world (Bocquet-Appel, 2002; Bocquet-Appel and Naji, 2006; Guerrero et al., 2008; Kohler et al., 2008) as an indirect measure of fertility. It has also been assumed that increased population size and density, combined with a new way of living (changes in diet, increased labor demands, sedentary lifestyle, proximity to domestic animals and poor hygienic conditions increasing the probability of various kinds of infections) resulted in an overall decline in health (Cohen and Armelagos, 1984; Cohen and Crane-Kramer, 2007; Bocquet-Appel, 2008; Cohen, 2008; Stock and Pinhasi, 2011). There is ample bioarchaeological evidence for the Neolithic health decline all over Europe (Jarosova and Dockalova, 2008; Wittwer-Backofen and Tomo, 2008; Papathanasiou, 2011; Stock and Pinhasi, 2011; Ash et al., 2016; Jovanović, 2017). Taken together, the increase in fertility and the health decline that resulted in increased mortality are the main two processes defining and structuring the Neolithic demographic transition (Bocquet-Appel, 2011). Both increased fertility and increased mortality should reflect themselves in the bioarchaeological record as an increase in various kinds of biological stress. However, so far there has not been any direct or independent evidence for the hypothesized increase of fertility. The proportion of subadults in skeletal assemblages is indeed more sensitive to changes in fertility than mortality (Sattenspiel and Harpending, 1983; McCaa, 2002) but mortality and cultural choices influence the age structure of skeletal populations. Therefore, the
proportion of subadults is a rather imprecise proxy of fertility. The crucial methodological problem is to find a way to measure fertility in a more direct way.

In this study we use the tooth cementum annulation method (TCA) to investigate physiological stress before and during the Neolithic demographic transition, as events of physiological stress are reflected in tooth cementum at an almost annual resolution. Moreover, we tentatively suggest that this increase can be seen as the first direct and independent evidence for the increase of fertility in the Neolithic, as pregnancies are the major etiological factor behind the stress proxy that we are using.

We are focusing our investigation on the region of the Central Balkans, where both Mesolithic and Neolithic cultures have been documented in the archaeological record. In the Central Balkans, the Mesolithic is primarily represented by communities that lived in the microregion of the Danube Gorges from the beginning of the Holocene around 9700 BC to around 5500 BC, subsisting on the aquatic resources and wild game (Borić, 2011). The first farmers arrived in the region around 6200 BC as the Neolithic way of life expanded from Greece towards the rest of the continent (Whittle et al., 2002; Porčić et al. in press). The earliest Central Balkan Neolithic is represented by the Starčevo culture that lasted until 5400/5350 BC (Garašanin, 1982; Porčić et al. in press). The arrival of the first farmers in the Central Balkans was followed by a relatively rapid population growth (Porčić et al. in press). Samples from the Mesolithic period presented in this study originate from a series of sites excavated in the Danube Gorges. The Neolithic samples originate from all Central Balkan Early Neolithic sites where human osteological material suitable for the TCA analysis was available, covering the large proportion of the Starčevo culture area (Fig. 1).

1.1. Tooth cementum

Tooth cementum is a mineralized tissue which covers the tooth root. Acellular extrinsic fiber cementum (AEFC) is located around the cervical third of each deciduous and permanent tooth. It shows a continuous appositional growth throughout life and is supporting the anchoring of the tooth in the alveolar bone (Kagerer and Grupe, 2001; Schröder, 2000) (Fig. 2). The tooth cementum preserves, like other mineralized tissues such as bone, enamel, dentine, incremental structures that are related to repeated physiological cycles (Dean, 2000) and can therefore be interpreted in a similar way as tree growth rings (Hogg, 2018). Each
pair of cementum rings (one brighter and one darker) forms an Incremental line (IL) and represents one year of life (Lieberman, 1994; Wedel, 2007). The Tooth cementum annulation (TCA) is mainly used as a method for age determination. It is considered that the formation of the ILs occurs under endogenous cycles and environmental factors related to seasons (Lieberman, 1993, 1994; Grue and Jensen, 1979), resulting in variation in terms of IL width and contrast. Irregular ILs (that could appear broader and/or more opaque) derive from physiological stressors that impact normal matrix formation, which are correlated to events such as pregnancies, birth, weaning, renal disease, bone trauma, climate and lifestyle changes (Macho et al., 1996; Simpson, 1999; Dirks et al., 2002; Schwartz et al., 2006; Bromage et al., 2011; Kagerer and Grupe, 2001; Künzie and Wittwer-Backofen, 2008; Cerrito et al., 2020). It has been known that physiologically demanding events such as pregnancy and lactation have multifold effects on the skeleton, such as significantly changed bone metabolic status (Miyamoto et al., 2019). Previous studies were able to show that pregnancies were in fact datable by the ILs and that the first trimester leaves marks in the tooth cementum (Kagerer and Grupe, 2001; Cerrito et al., 2020). Although the methodology for the determination of IL is still not standardized, recent publications have dealt with this issue (Mani-Caplazi, 2019). Therefore, teeth give, even after thousands of years, an incomparable insight into the life of a deceased person and let us learn something about their life, the stress they had to endure and possibly the number of descendants they had.

In this study, we used the TCA method by measuring irregular ILs to quantify prehistoric physiological stress from the perspective of the
differences in health and general stress between the Mesolithic and Neolithic populations. Our sample consists of 46 teeth from 46 individuals (sex and age structure are presented in Table 1). All the samples are from secure archaeological contexts, excavated at sites located in the Central Balkan area. Fifteen of them have published radiocarbon dates, and additional seventeen samples were AMS dated for the purpose of this study (Supplementary Table 1). The rest of the samples were determined to be either Mesolithic or Neolithic based on the relative or absolute dating of the entire site and the burial position. Sex of the individuals used in this study is based on previous macroscopic analyses on the skeletal material and aDNA analyses where available (Rajić Sikanjić and Premužić, 2015; Hofmanová, 2016; Jovanović, 2017; Jovanović et al.; Mathieson et al., 2018).

Our hypothesis is that the Neolithic individuals will on average have more physiological stress episodes than Mesolithic individuals. More specifically, a significantly higher number of tooth cementum stress lines should be present in Neolithic females, as a reflection of increased fertility, as well as the general increase in stress levels due to a Neolithic way of life (Bocquet-Appel, 2011).

2. Materials and methods

2.1. The sample

Samples are coming from human remains that originate from several Mesolithic and Neolithic sites from the territory of the Central Balkans (Supplementary table 1). The temporal range of the Mesolithic sample is rather wide (from ~9700 to ~6160 BC) but it consists exclusively of the individuals from the microregion of the Danube Gorges where a continuity of the Mesolithic lifestyle was documented from the beginning of the Holocene (Borić, 2011). Therefore, a Mesolithic individual from ~7500 BC lived a similar life as an individual from ~6500 BC. As for the Neolithic sample, some of the Neolithic individuals (N = 15) come from the Danube Gorges, therefore, there is full geographical continuity, whereas others come from the rest of the Central Balkans area. The Central Balkans is a relatively small region, with the distance between the farthermost sampled sites not exceeding 300 km. The climatic fluctuations were not great in the Holocene and all samples come from the same climate zone. Therefore, all these individuals on average experienced the same temperate continental climate.

For the purpose of this study 90 single-rooted teeth representing both female and male individuals of different ages have been sampled. Forty-six of those produced sections that met the criteria we have set to be either Mesolithic or Neolithic based on the relative or absolute dating of the entire site and the burial position. Sex of the individuals used in this study is based on previous macroscopic analyses on the skeletal material and aDNA analyses where available (Rajić Sikanjić and Premužić, 2015; Hofmanová, 2016; Jovanović, 2017; Jovanović et al.; Mathieson et al., 2018).

2.2. Sample preparation and age-at-death determination

The laboratory work was conducted at the Institute for Biological Anthropology, University clinic, Freiburg i.B., Germany. The general protocol for the preparation of teeth was based on previous studies conducted at this institute (Wittwer-Backofen, 2012; Penezić et al., 2019). Due to the fragility of the specimens, they were embedded into Biodur epoxy resin (Biodur E12 resin with hardener E1 in the ratio 100:28). The middle third of each tooth was cut cross-sectionally by a Leica SP 1600 (rotating diamond) microtome. Thickness of each cut was 80 μm. This procedure resulted in 4–20 cuts per tooth. A transmission light microscope Leica DM RXA 2 with magnifications of 20 × and 40 × was used for the documentation of all sections. Each section was observed by the first author. A digital tubus camera Leica DC 250 was used to record and photograph all regions of interest on each cut of each tooth. One representative photograph per cut was selected for the age at death calculations. Number of pairs of light and dark cementum rings, forming one IL, was counted per photo. The age at death was calculated by using the average number of IL per tooth, to which the sex specific average age of tooth eruption for the respective tooth type (Adler, 1967) was added.

2.3. Method for stress layer determination

From the representative photographs used for the age at death calculations, three photos (originating from three different sections) were selected and used for stress layer determination. Usually, the selected photographs had the same/similar number of counted ILs. The thickness of the cementum band (the whole AEFC deposition) was measured at four positions at each photo, and the average value was used. The average thickness of the cementum band was divided by the number of counted ILs for that specific cut, providing the average thickness of ILs. ILs that are observed to be broader and more opaque and appear so on the most part of the photograph were measured. If the thickness was greater than the average incremental line thickness for the specific cut, this ILs was described as a stress layer. Only if the stress layer occurred at the corresponding position within the cementum band on at least two out of three cuts, it was considered a verified stress layer and used towards the final count for the specific tooth. Illustrations for the verified stress layers are presented in Fig. 4. This method relies on the observer’s pre-selection of stress layers (where only ILs that appear irregular to the observer are measured). All samples and photos were analyzed under their lab number, until the final statistical analysis. This was done in order to avoid possible preconceptions about the sex and the period that the samples originate and to avoid any possible expectations influencing the final results.

A small-scale pilot study compared two different methods for stress layer determination (Penezić et al., 2019). The selection of photographs for stress layer determination was conducted as described in this study, as well as AEFC cement band measurements. As for the stress layer determination, the first method was as described in this study, while the second method consisted of measuring each IL with the Leica software measuring tool. For each section (represented by one photograph) average width of ILs and the corresponding standard deviation was calculated. All measured ILs that had the value greater than the average + 1 standard deviation were defined as stress layers. The main criterion for stress layer determination by applying this method was their differing width. Comparison of these two methods showed the mean value of 93.6 percent of matching stress layers per tooth.

| Table 1 | Sex and age structure of the Mesolithic and Neolithic populations. |
|---------|---------------------------------------------------------------|
|         | Female Age | Male Age |
|---------|-------------|----------|
|         | Mean       | Minimum  | Maximum |
| Mesolithic | 53.28      | 28.72    | 69.99    | 52.01      | 42.23    | 67.94    |
| Neolithic  | 45.54      | 21.56    | 68.82    | 39.16      | 29.56    | 47.45    |
2.4. Calculation of individual burden of stress

For each individual in our sample we calculated the number of stress layers per year of life (after tooth eruption) by dividing the number of stress layers (as determined by the protocol presented above) with the total number of TCA ILs (as in Penezić et al., 2019). This standardization is necessary in order to make individuals with different ages at death comparable in terms of the number of stress layers (as it is more likely for individuals who lived longer to experience more stress).

2.5. Statistical analysis

We formally test our hypothesis by statistically comparing the number of stress layers per year of life between Mesolithic and Neolithic females, and Mesolithic and Neolithic males. We use the Mann-Whitney test with the chronological phase (Mesolithic vs. Neolithic) as a grouping variable and the number of stress layers per year of life as a dependent variable. We perform separate Mann-Whitney tests for females and males, implemented in the IBM SPSS Statistics 23 software.

3. Results

The stress layers were identified in all samples, and they occur in both females and males, as well as in both Mesolithic and Neolithic periods. The range of the stress lines per person is 1–7, and the earliest occurrence of a stress line could be seen at the age 8, where the latest one was identified at the age of 60. We grouped the samples per sex as well as per period in order to explore the differences. For additional 17 samples AMS dates have been obtained within our study (Supplementary table 1).

As predicted by our hypothesis, the Neolithic females had more stress layers on average than the Mesolithic females. They also had greater individual variability in the number of stress layers, whereas the situation is exactly the opposite for males both in the Mesolithic as well as in the Neolithic group (Table 2, Figs. 5–6).

In order to quantify and compare the stressful events per lifetime per person, we calculated the distribution of stress layers per year of life. The difference in the distribution of stress layers per year of life between the Mesolithic and Neolithic females is statistically significant (U = 61, exact one-tailed p = 0.001). On the other hand, the difference between Mesolithic and Neolithic males is not statistically significant (U = 10, exact one-tailed p = 0.214). Therefore, we can conclude that Neolithic women indeed had a higher number of physiological stress episodes than Mesolithic women. For men, the levels of stress were practically the same. However, there are also no statistically significant differences between males and females in general (U = 173, exact one-tailed p = 0.315). The boxplot in Fig. 5 suggests that the variance in the Neolithic female sample is greater than in the Mesolithic female sample, but this difference is not statistically significant (Levene’s test: F = 1.867, p = 0.181).

![Fig. 4. a) Representative cut from grave 2 at Ervenica Poljski Jarak with counted 48 ILs and determined 4 stress layers (indicated by arrows), b) Representative cut from grave 32b at Lepenski Vir with counted 32 ILs and determined 5 stress layers (indicated by arrows).](image-url)
and Neolithic individuals. Research convincingly shows that the shape of the distribution of ASF births given by a woman in a particular age category. Cross-cultural conditions (renal disease, trauma or environmental conditions) reflect pregnancies (Kagerer and Grupe, 2001; Künzie and Wittwer-Backofen, 2008; Cerrito et al., 2020).

In order to explore whether stress layers mostly reflect pregnancies in the female sample in contrast to the male sample where they reflect unspecified stress, we compared the age distributions of stress layers between males and females. The age distribution is a frequency of stress layers in five-year age categories based on aggregated data from individuals, and we compare it between the female and male sample. We first establish the number of individuals who lived through a certain age category by looking at the cumulative distribution of ages. For example, if there are three individuals who lived 24, 28 and 34 years, respectively, there would be 3 persons in the age category of 20–24.99 years, 2 in the 25–29.99 category and 1 person in the 30–34.99 category. We calculate the average number of stress layers per person in a particular age category by dividing the number of stress layers with the number of individuals who lived through this age category. We will refer to this index as the age specific stress rate (ASS). If the cause of stress is pregnancy, this measure is equivalent to the age specific fertility rate (ASF), a standard demographic parameter. ASF represents the average number of births given by a woman in a particular age category. Cross-cultural research convincingly shows that the shape of the distribution of ASF rates is almost invariable across different preindustrial communities, so that we can speak of a general preindustrial pattern of the ASF in humans (Chamberlain, 2006; Handwerker, 1983; Ellison et al., 2012).

In this general pattern the ASF values increase from menarche, reach maximum between 20 and 30 years, and decline towards the age of the menopause (45–50 years) (Fig. 7). The idea is to compare the shape of the ASS distribution based on the TCA data to the universal ASF distribution pattern characteristic of the preindustrial communities. If the TCA stress layers reflect mostly pregnancies in females and some unspecified stress in males (e.g. bone breakage or other trauma, renal disease) the shapes of the ASS distributions should be different for males and females. Moreover, the female distribution of the ASS should be similar to the preindustrial pattern of the ASF if most of the stress layers in females are caused by pregnancy.

In our sample, the distributions of ASS are markedly different for males and females (Fig. 7). For males, the stress is concentrated in the first two decades of life, the ASS peaks at 20–24.99 years and then relatively quickly plummets to zero as the 40–44.99 category is reached. For females, the pattern is more complex. For the age span between birth and 50 years, the ASS loosely resembles the general fertility pattern of preindustrial communities, but after 50 years of age the rate increases again, peaking at 55–59.99 years. The increase in frequency of stress after 50 years of age in females might be related to higher susceptibility to illness as a result of old age. No such increase is seen in the male subsample, but there are only two male individuals older than 50 years, therefore, this lack of signal may be due to sampling error. These results suggest that the etiology of the physiological stress was different for males and females.

The facts that (1) pregnancies are one of the leading causes of the TCA stress layers and (2) the shape of the distribution of stress in females does resemble the general age specific fertility pattern in humans at least for the fertile period of life, and (3) the increase in the number of stress layers from the Mesolithic to the Neolithic is detected only in females, provide basis to conclude that our results can be interpreted as demonstrating the increase in the number of pregnancies and fertility associated with the Neolithic Demographic Transition. The marked increase in fertility during the Neolithic would be consistent with the relatively high growth rate estimates of the Central Balkan Neolithic population (Porčić et al. in press). This does not mean that each stress layer in females is caused by pregnancy - we can be certain that stress layers occurring after 50 years of age are not. We only suggest that the number of pregnancies is the major contributing factor to the pattern that we observe in the data as it is difficult to imagine why the frequency of renal disease or physical trauma, as other potential causes of physical stress, would change for the Neolithic females in contrast to the Mesolithic females. As for the potential environmental factors that could influence the occurrence of stress markers, they would influence both males and females equally.

It is interesting to note that there are no significant differences in the frequency of stress between males and females. One might argue that this fact goes against the interpretation of the observed patterns as evidence of increased fertility. However, this does not have to be the case. We have already shown that the age distribution of stress layers is different for males and females - in the male subsample, the stress is concentrated in the late teens and early twenties. Based on this, we would hypothesize that most of the stress observed in males has to do with the fact that men are more exposed to physical trauma due to heavy workloads, higher mobility (especially in the Mesolithic) and interpersonal violence (Roksandić et al., 2006). This hypothesis should be

### Table 2

Descriptive statistics for the number of stress layers per year of life (after tooth eruption).

|           | Mean | Median | St.Dev. | N  | Mean | Median | St.Dev. | N  |
|-----------|------|--------|---------|----|------|--------|---------|----|
| Mesolithic | .0541| .0472  | .0366   | 15 | .0910| .0773  | .0333   | 6  |
| Neolithic | .1031| .0942  | .0538   | 20 | .0686| .0732  | .0254   | 5  |

![Fig. 5. Boxplot of the number of stress layers per year of life of the Mesolithic and Neolithic individuals.](image-url)

4. Discussion

We demonstrated that the Neolithic females had significantly more stress layers than the Mesolithic females, whereas this pattern is not found in males. Clinical studies suggest that stress layers in tooth cementum may inter alia (renal disease, trauma or environmental conditions) reflect pregnancies (Kagerer and Grupe, 2001; Künzie and Wittwer-Backofen, 2008; Cerrito et al., 2020).

In order to explore whether stress layers mostly reflect pregnancies in the female sample in contrast to the male sample where they reflect unspecified stress, we compared the age distributions of stress layers between males and females. The age distribution is a frequency of stress layers in five-year age categories based on aggregated data from individuals, and we compare it between the female and male sample. We first establish the number of individuals who lived through a certain age category by looking at the cumulative distribution of ages. For example, if there are three individuals who lived 24, 28 and 34 years, respectively, there would be 3 persons in the age category of 20–24.99 years, 2 in the 25–29.99 category and 1 person in the 30–34.99 category. We calculate the average number of stress layers per person in particular age categories by dividing the number of stress layers with the number of individuals who lived through this age category. We will refer to this index as the age specific stress rate (ASS). If the cause of stress is pregnancy, this measure is equivalent to the age specific fertility rate (ASF), a standard demographic parameter. ASF represents the average number of births given by a woman in a particular age category. Cross-cultural research convincingly shows that the shape of the distribution of ASF rates is almost invariable across different preindustrial communities, so that we can speak of a general preindustrial pattern of the ASF in humans (Chamberlain, 2006; Handwerker, 1983; Ellison et al., 2012).

In this general pattern the ASF values increase from menarche, reach maximum between 20 and 30 years, and decline towards the age of the menopause (45–50 years) (Fig. 7). The idea is to compare the shape of the ASS distribution based on the TCA data to the universal ASF distribution pattern characteristic of the preindustrial communities. If the TCA stress layers reflect mostly pregnancies in females and some unspecified stress in males (e.g. bone breakage or other trauma, renal disease) the shapes of the ASS distributions should be different for males and females. Moreover, the female distribution of the ASS should be similar to the preindustrial pattern of the ASF if most of the stress layers in females are caused by pregnancy.

In our sample, the distributions of ASS are markedly different for males and females (Fig. 7). For males, the stress is concentrated in the first two decades of life, the ASS peaks at 20–24.99 years and then relatively quickly plummets to zero as the 40–44.99 category is reached. For females, the pattern is more complex. For the age span between birth and 50 years, the ASS loosely resembles the general fertility pattern of preindustrial communities, but after 50 years of age the rate increases again, peaking at 55–59.99 years. The increase in frequency of stress after 50 years of age in females might be related to higher susceptibility to illness as a result of old age. No such increase is seen in the male subsample, but there are only two male individuals older than 50 years, therefore, this lack of signal may be due to sampling error. These results suggest that the etiology of the physiological stress was different for males and females.

The facts that (1) pregnancies are one of the leading causes of the TCA stress layers and (2) the shape of the distribution of stress in females does resemble the general age specific fertility pattern in humans at least for the fertile period of life, and (3) the increase in the number of stress layers from the Mesolithic to the Neolithic is detected only in females, provide basis to conclude that our results can be interpreted as demonstrating the increase in the number of pregnancies and fertility associated with the Neolithic Demographic Transition. The marked increase in fertility during the Neolithic would be consistent with the relatively high growth rate estimates of the Central Balkan Neolithic population (Porčić et al. in press). This does not mean that each stress layer in females is caused by pregnancy - we can be certain that stress layers occurring after 50 years of age are not. We only suggest that the number of pregnancies is the major contributing factor to the pattern that we observe in the data as it is difficult to imagine why the frequency of renal disease or physical trauma, as other potential causes of physical stress, would change for the Neolithic females in contrast to the Mesolithic females. As for the potential environmental factors that could influence the occurrence of stress markers, they would influence both males and females equally.

It is interesting to note that there are no significant differences in the frequency of stress between males and females. One might argue that this fact goes against the interpretation of the observed patterns as evidence of increased fertility. However, this does not have to be the case. We have already shown that the age distribution of stress layers is different for males and females - in the male subsample, the stress is concentrated in the late teens and early twenties. Based on this, we would hypothesize that most of the stress observed in males has to do with the fact that men are more exposed to physical trauma due to heavy workloads, higher mobility (especially in the Mesolithic) and interpersonal violence (Roksandić et al., 2006). This hypothesis should be
Fig. 6. Distribution of the number of stress layers per year of life by sex and chronology.

Fig. 7. Comparison of the age-specific stress rate (ASS) for the female and male subsamples (left upper and lower panel), to the age-specific fertility rates (ASFR) for three preindustrial populations (redrawn from Chamberlain, 2006: Fig. 2.7) (right panel).
5. Conclusion

Our results support the hypothesis of increased physiological stress in prehistoric farmer populations. The differential pattern between sexes may be tentatively interpreted as evidence for the increase of female fertility in the Neolithic period - that the increase in the number of births was a major (but not the only) contributor to the increase in stress during the Neolithic transition. If this interpretation is true, our results would provide direct empirical evidence in favor of the general Agricultural demographic transition theory where increase of fertility is postulated as the main driver of farming population expansion (Bocquet-Appel, 2011). The method that we used has a wide bio-archaeological potential, as it allows the researchers to get a more direct insight into physiological stress in past populations. Perhaps the greatest potential of the method is to explore a range of paleodemographic and social questions involving births and motherhood, but this avenue of interpretation requires further methodological research in order to formulate criteria for distinguishing pregnancies from other causes of stress.

CRediT authorship contribution statement

Kristina Penezić: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. Marko Poreč: Conceptualization, Formal analysis, Writing - original draft. Petra Kathrin Urban: Methodology, Writing - original draft. Ursula Wittwer-Backofen: Methodology. Sofija Stanojević: Conceptualization, Funding acquisition.

Declarations of competing interest

None.

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Appendix A. Supplementary data

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