Article

Children's Cortisol and Cell-Free DNA Trajectories in Relation to Sedentary Behavior and Physical Activity in School: A Longitudinal Analysis

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Abstract: Education outside the classroom (EOtC) can be beneficial for students. The relationship between biological stress markers and sedentary behavior (SB) plus physical activity (PA) is insufficiently evaluated in school settings. This exploratory study aims to evaluate the association between students' cortisol, plus circulating cell-free deoxyribonucleic acid (cfDNA) levels, and their SB, light PA (LPA) and moderate-to-vigorous PA (MVPA) during outdoor and indoor classes in different seasons. We assessed data from an education outside the classroom (EOtC) program (n = 48; intervention group [IG], n = 37; control group [CG], n = 11). We sampled data on 3 school-days in three seasons (fall, spring, and summer) in normal teaching indoors (CG) and outdoor lessons (IG) in the forest. SB and PA were evaluated by accelerometry, and cortisol and cfDNA levels by saliva samples. The compositional data analysis approach analyzed SB and PA. Fitted Bayesian hierarchical linear models evaluated the association between cortisol and cfDNA, and SB/LPA/MVPA. A steady decline of cortisol in the outdoor setting is associated with relatively high levels of LPA. SB and MVPA tended to exhibit a similar effect in the indoor setting. CfDNA is positively associated with a relatively high amount of SB in the IG, the same association is likely for LPA and MVPA in both groups. LPA seems to support a healthy cortisol decrease in children during outdoor lessons. The relevance of SB/PA as a composition in relation to students stress response in school should be emphasized. This study facilitates the formulation of straightforward and directed hypotheses for further research.

Keywords: cortisol; cfDNA; physical activity; outdoor education; stress; health, Baysian inference

1. Introduction

As the upsurge in the worldwide prevalence of overweight and obesity in children is anticipated to reach 9.1% in 2020 [1], a high proportion of children do not reach the recommended levels of physical activity (PA) [2-3], and suffer from mental disorders [4]. In contrast, PA during childhood correlates with the development of active lifestyles in later life and, thus, with positive effects on health and prevention of common diseases [5]. However, chronic stressful events could exert adverse impacts on brain development and result in major mental health-related problems in later life [6-7]. At present, children spend a substantial share of their waking hours in school and the opportunities for being physically active during leisure time and free-play potentially decline from kindergarten over the primary school to secondary school because of longer school days. In turn, the compulsory school system in western countries offers excellent opportunities to reach every child and adolescent with specific interventions toward more PA and mental wellbeing. The present paper aims to address these topics by investigating the relation between biological stress responses and physical activity in students taught in different school settings. Here, we extend our original investigation [8] by increasing our set of dependent variables and introducing cell free DNA (cfDNA) as an innovative biological marker, sedentary behavior (SB) and light physical activity (LPA) as more differentiated measures of physical activity and by applying sophisticated statistical models to better describe relations between our measures.

Recent studies have focused on exploring the construct of "stress" and its potential negative association with health [9]. In fact, an individual's physiological and psychological response, assessed by different stress biomarkers or questionnaire items, could be correlated with several positively, as well as negatively, connoted stimuli. Koolhaas et al. [9] argued that the term "stress" should be restricted to situations of uncontrollability or unpredictability of stimuli which however, must be restricted to "psychological stress" and is not true for so called "physical stress"[10-11], which can be defined as a loss of homeostasis induced by physical not psychological conditions. Examples of such uncontrollable situations in school are examinations, testimonials, increased mental loads or prolonged social pressure [12-13]. Such stressors lead to an interruption of the regular circadian cortisol rhythm. In addition, the existence and relevance of a normal diurnal cortisol rhythm with high levels of cortisol in the morning and a steady decline until evening has been widely investigated [14-16]. Furthermore, several external stimuli could be involved in the disturbance of a normal diurnal cortisol rhythm. For instance, light pollution during night time, continuous changes in waking hour schedules, heavy cognitive, or psychological loads can affect a normal cortisol diurnal rhythm. Moreover, a dysfunctionality in the hypothalamic pituitary adrenocortical axis as one primary biological stress system plays a crucial role. In a recent systematic literature review and meta-analysis, Adam et al. [17] reported that a chronic abnormal flat diurnal cortisol rhythm correlated with poor mental and physical health symptoms for various populations. Other experimental studies [18-19] evaluated the association between cortisol levels and PA, with a particular focus on different PA intensities, as well as the diurnal cortisol rhythm; these studies revealed that high PA intensities ranging 60%–80% the maximal oxygen uptake (VO_2 max) [18] or only 80% VO_2 max [19] for, at least, 30 min resulted in statistically significant higher cortisol levels compared with resting control situations. Interestingly, participants' cortisol levels decreased, although not statistically significant, not only in the resting control groups (CG) but also during low PA intensities of 40% VO_2 max. These studies illustrate the potential impact of PA on cortisol levels.

Besides the well-established but also critically discussed stress marker cortisol [20], the circulating cell-free deoxyribonucleic acid (cfDNA) has garnered more importance as a potential physiological stress marker. Different mechanisms can result in the release of the cfDNA into the human plasma. While an increase in cfDNA levels because of classic cell-death mechanisms would take several hours, or even days, other more rapid mechanisms are related to exercise. Based on plasma samples, the cfDNA is a well-established indicator of the activation of the innate immunity. Various studies have revealed that the innate immunity could be activated by both psychologically [21-22] and physiologically [23-27] stressful situations. In particular, the cfDNA has been proven to be highly sensitive to physical exercise as a stressor (see [27] for review). Reportedly, the cfDNA

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increased with moderate PA below the level of the aerobic–anaerobic transition [26, 28]. Regarding psychological stress, little is known about the reactivity of cfDNA concentrations. To date, only one study has reported that lowering psychological stress in women treated for infertility reduces the plasma cfDNA concentration, a notion that is principally in line with the concept of a stress-associated, sensitive proinflammatory marker; however, the concept warrants further investigation [29]. Most research on the cfDNA is restricted to plasma samples and controlled laboratory settings. However, a study has reported that the cfDNA in the saliva and serum possess a similar half-life time and both follow a first-order clearance model [30]. Furthermore, in both body fluids, the cfDNA seems to be predominantly released by cells of the hematopoietic lineage [25, 31]. To the best of our knowledge, no research has investigated the association between cfDNA levels based on salivary samples and exercise in an experimental setting, at least, in schools. As we have outlined above, the secretion and metabolization of both cortisol and cfDNA in humans are related to SB and PA. Thus, the comparison of both cortisol and cfDNA in relation to students' relative levels of SB and PA is a promising approach to investigate students' biological stress response in different school settings.

As outlined earlier, being physically active is highly essential for one's health status [3]. In addition, PA and SB are potential confounders for cortisol and cfDNA. With a focus on public health, SB has been discussed in the last decade. The authors of a recent review [32] suggested that high values in sedentary time correlated with an increased risk of cardio-metabolic disease, decreased fitness, self-esteem, academic achievement, and pro-social behavior for children and adolescent [32]. Very obviously there is also a relation between SB and mental health, particularly depression [33]. However, the reported evidence is often limited because of a lack of causality, and therefore, warranting further investigation [34].

A great responsibility for children's PA and health could be assigned to educational institutions and their schedules. Apparently, students' time in school and its environment play a crucial role. Typically, natural green environments (NGE) and PA in NGE seem to be beneficial for promoting children's PA and health [35-37]. In a recent systematic literature review [38], we assessed the effects of regular compulsory school and curriculum-based education outside the classroom (EOtC, [39]) programs, focusing on students' health, PA, social, and learning dimensions. EOtC often takes place in both NGE and cultural settings. Unfortunately, the methodological quality of the 13 included studies was mostly moderate or low. In addition, students seem to benefit regarding learning and social dimensions. However, only one study reported improved mental health status of boys [40] and two studies [41-42] reported higher PA levels than those during days with EOtC compared with regular school days. Moreover, a recent large-scale study [43] on EOtC reported that the MVPA levels were significantly higher during EOtC compared with regular school days. However, the codependency among students SB, LPA, and MVPA levels remained unclear in this study. Overall, the existing knowledge on effects of EOtC is limited, despite the mentioned potentials of this type of teaching setting.

In our recent publication [8], we compared the cortisol levels of students taught by applying an outdoor curriculum in the forest with children taught in the standard school setting; We were primarily interested in assessing the effect of outdoor teaching on children's normal diurnal cortisol rhythms. We reported that students in the intervention group (IG) exhibited a steady decline of cortisol levels during EOtC, whereas no such effect was observed in students in the CG during regular school days; in fact, the effect was independent of students MVPA levels. However, we could not entirely elucidate the differences in students' cortisol levels. We believe that the partial secondary exploitation of the data is justified by the new knowledge gained, as we analyzed the cortisol and cfDNA values concerning the compositional nature of SB and PA.

This exploratory, longitudinal analysis aims to evaluate the association between students' cortisol and cfDNA levels and their SB, LPA, and MVPA in outdoor and indoor classroom environments.

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2. Results

2.1. Descriptive Statistics

Table 1 shows the descriptive statistics for the variables SB, LPA, and MVPA, separated for seasons and groups. We observed no evident differences between the arithmetic mean and the compositional mean in this study. Most apparent differences were observed in higher relative means of SB for the CG compared to the IG and lower relative means of MVPA for the CG compared to the IG. We neitherobserved any evident differences in seasons and the relative means of LPA. Table 2 presents the variability in the data of students' behavior in a variation matrix, segregated by seasons and groups; values close to zero in the table implicate that both involved behaviors are highly codependent. We observed the highest log-ratio variances for both groups in MVPA, except for the log-ratios of SB/LPA in spring and summer. Thus, the codependency for SB/LPA is most often stronger compared to SB/MVPA and LPA/MVPA. The pair-wise log ratios are lower for the CG compared with the IG, except for the codependencies of SB/LPA and SB/MVPA in fall and SB/MVPA in spring. Figure 1 displays a compositional analysis of the relative importance of students' mean time in SB, LPA, and MVPA separated by groups and seasons, with respect to the overall mean time composition in each behavior. In addition, the ternary plots S1 and S2 present the analysis on concordance with the results (Supplementary Material section Results). Regarding the overall mean time composition, the relative amount of SB in fall and summer is high for the CG and low for the IG; we observed no difference in spring. In addition, the relative amount of LPA is contrary to seasons and groups. Moreover, we almost observed no difference in fall and summer, whereas the relative amount in spring was high in the CG and low in the IG. Furthermore, the relative amount of MVPA in fall, spring, and summer was low for the CG and high for the IG. Of note, the descriptive results of the arithmetic and compositional mean for SB, LPA, and MVPA (Table 1) accord with the ternary plot S2.

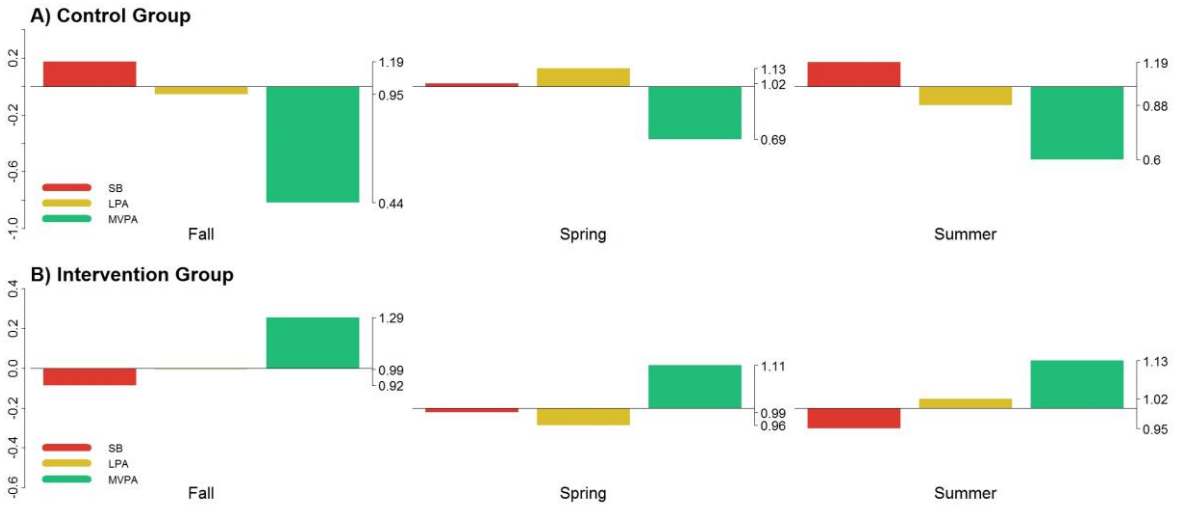

Figure 1. The compositional analysis of the relative importance of students' mean time spent in sedentary behavior (SB), light physical activity (LPA), and moderate-to-vigorours physical activity (MVPA) with respect to the overall mean time composition. Panel A) displays the control group; panel B) displays the intervention group; both panels are segregated by seasons (fall; spring; summer). The left axis, the log-ratio value; the right axis, the proportion relative to the mean composition (e.g., 1.29 means a proportion higher by 29%).

2.2. Association between Cortisol, cfDNA, SB, LPA, MVPA

We fitted different Bayesian hierarchical linear models (BHLMs) to assess the possible impact of the relative amounts of SB, LPA, and MVPA on students' cortisol and cfDNA levels. The BHLMs for cortisol and cfDNA differed between the applied indices peak reactivity (PR) and the area under the curve with respect to increase (AUCi). Regarding models with AUCi values in the covariates group (CG/IG), gender (female/male) and season (fall/spring/summer) were included. Regarding models with PR values, the covariate time point (midmorning/noon) was additionally included. In addition, we evaluated the model fit by means of the deviance information criterion (DIC). The convergence of the Markov chains were investigated by posterior predictive checks (cf. Figures S9–S12 for details; only the results for the respective best fitting BHLMs are presented).

2.2.1. Association between SB and PA in the cortisol PR and AUCi

According to the Markov chain Monte Carlo (MCMC) posterior distributions (cf. Figure 2 and Table 3 for summary and Tables A1–A2 for details), we observed a strong negative association in the IG for the relative amounts of LPA on the cortisol PR levels (mean = –0.728; lower confidence interval [CI]: –1.268; upper CI: −0.190). In the CG, tendencies of a negative association were noted between SB and MVPA in the cortisol PR. Regarding cortisol AUCi, we observed the likelihood of a negative association in the IG for LPA. In the CG, however, a tendency was observed for SB in cortisol AUCi. Considering both mean values and CIs, the IG exhibited stronger associations compared to the CG.

2.2.2. Association between SB and PA in the cfDNA PR and AUCi

Compared with the cortisol PR and AUCi, the results of the cfDNA PR and AUCi were different. In the IG, we observed the likelihood of a positive association between SB and MVPA in the cfDNA PR. In fact, a strong positive association was noted in the IG for SB (mean, 1.285; lower CI: 0.390; upper CI: 2.191) and tendencies of a positive association of LPA and MVPA in the cfDNA AUCi. In the CG, tendencies of a positive association were found for LPA and MVPA in the cfDNA AUCi (cf. Figure 2 and Table 3 for summary and Tables A3–A4 for details).

3. Discussion

We concucted the present study to provide an update on the associations between students' cortisol levels and their physical activity as reflected in the measures of SB, LPA and MVPA as well as associations between students' cfDNA levels and their SB, LPA and MVPA during outdoor and indoor classes. While interpreting the results of this study, one must consider the character of this exploratory study: the school setting in which both (a) the number of available participants is low because of the situation of EOtC in Germany, and (2) the number of possibly uncontrolled confounders is high because of the real-world scenario. However, we believe that our study can provide valuable insights into the EOtC research, health promotion in schools, and the assessment and analysis of cortisol, cfDNA, SB, and PA in the educational setting. In our previous study [8], we reported a statistically significant difference in the measured cortisol levels between the CG and IG; regular teaching in the forest correlated with a lower cortisol secretion at noon compared with the standard indoor teaching, and this association was independent of students' MVPA levels. Considering the compositional nature and, thus, the codependency of students' SB, LPA, and MVPA, we elucidated students' cortisol values during school time in this study. Furthermore, we compared those results with associations between students' cfDNA levels and their SB and PA.

According to these analysis, the results of cortisol and cfDNA regarding the peak reactivity and AUCi are diverse. First, we could partially confirm our previously reported results [8] reflecting their independence of the analysis methodology, as students' cortisol levels were not affected by the relative amounts of MVPA in the IG. However, in the CG, the relative amount of

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MVPA is more likely to exert a lowering effect on the cortisol PR; the more active the students were in MVPA levels, the more their cortisol levels seemed to decrease. Two experimental studies [18-19] reported that human behavoirs similar to SB and LPA correlated with declining cortisol levels, which is in concordance with a typical diurnal rhythm. The lowering effect of LPA on cortisol in the IG therefore corroborates Hill et al. [18] and VanBruggen et al. [19], although the specific PA

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Table 1. Descriptive characteristics of arithmetic and compositional mean for SB, LPA, and MVPA. Results are presented in percentage of mean time in each behavior on the respective school day, segregated by seasons and groups.

	Fall CG			Spring CG			Summer CG		
	SB	**LPA**	**MVPA**	**SB**	**LPA**	**MVPA**	**SB**	**LPA**	**MVPA**
Arithmetic mean (SD)	65.15 (7.28)	27.29 (6.62)	7.56 (2.90)	57.80 (10.48)	29.99 (5.14)	12.21 (7.68)	63.61 (12.02)	25.66 (9.11)	10.73 (4.32)
Compositional mean	65.79	27.01	7.19	58.65	30.50	10.85	65.18	24.81	10.01
	Fall IG			**Spring IG**			**Summer IG**		
	SB	**LPA**	**MVPA**	**SB**	**LPA**	**MVPA**	**SB**	**LPA**	**MVPA**
Arithmetic mean (SD)	50.19 (7.81)	28.21 (4.95)	21.59 (6.28)	56.03 (8.86)	25.68 (5.45)	18.29 (6.51)	51.78 (9.31)	28.74 (5.09)	19.48 (6.43)
Compositional mean	50.58	28.34	21.07	56.75	25.74	17.51	52.17	28.97	18.86

SB, sedentary behavior; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity; CG, control group; IG, intervention group; SD, standard deviation.

Table 2. The compositional variation matrix for time spent in SB, LPA, and MVPA, segregated by groups and seasons.

	Fall			Spring			Summer		
	SB	**LPA**	**MVPA**	**SB**	**LPA**	**MVPA**	**SB**	**LPA**	**MVPA**
	CG/IG	CG/IG	CG/IG	CG/IG	CG/IG	CG/IG	CG/IG	CG/IG	CG/IG
SB	0/0	0.112/0.124	0.238/0.211	0/0	0.145/0.164	0.395/0.393	0/0	0.070/0.215	0.079/0.502
LPA	0.112/0.124	0/0	0.253/0.166	0.145/0.164	0/0	0.043/0.125	0.070/0.215	0/0	0.045/0.085
MVPA	0.238/0.211	0.253/0.166	0/0	0.395/0.393	0.043/0.125	0/0	0.079/0.502	0.045/0.085	0/0

SB, sedentary behavior; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity; CG, control group; IG, intervention group.

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Table 3. The MCMC output of posterior probabilities. BHLMs 1–4: values are presented if the lower CI (25%) and upper CI (75%) do not overlap 0; respective values are bold.

Variable	**Model 1**	**Mean**	**SD**	**CI 2.5%**	**CI 25%**	**CI 50%**	**CI 75%**	**CI 97.5%**	**Rhat**	**n.eff**
SB (CG)	BHLM 1: cortisol PR	–0.215	0.152	–0.512	**–0.318**	**–0.213**	**–0.112**	0.085	1.001	7500
LPA (IG)	BHLM 1: cortisol PR	–0.728	0.271	**–1.268**	**–0.908**	**–0.726**	**–0.551**	**–0.190**	1.001	7500
MVPA (CG)	BHLM 1: cortisol PR	–0.499	0.524	–1.517	**–0.860**	**–0.501**	**–0.141**	0.527	1.001	5900
SB (CG)	BHLM 2: cortisol AUCi	–0.293	0.298	–0.880	**–0.494**	**–0.291**	**–0.095**	0.297	1.001	7100
LPA (IG)	BHLM 2: cortisol AUCi	–1.027	0.550	–2.112	**–1.403**	**–1.030**	**–0.661**	0.062	1.001	6700
SB (IG)	BHLM 3: cfDNA PR	0.242	0.289	–0.329	**0.049**	**0.246**	**0.435**	0.801	1.001	5400
MVPA (IG)	BHLM 3: cfDNA PR	0.839	0.636	–0.418	**0.416**	**0.839**	**1.269**	2.088	1.001	7500
SB (IG)	BHLM 4: cfDNA AUCi	1.285	0.464	**0.390**	**0.970**	**1.286**	**1.595**	**2.191**	1.001	7500
LPA (CG)	BHLM 4: cfDNA AUCi	1.643	0.877	–0.072	**1.058**	**1.652**	**2.232**	3.348	1.001	4300
LPA (IG)	BHLM 4: cfDNA AUCi	1.231	0.858	–0.455	**0.647**	**1.227**	**1.804**	2.899	1.001	5300
MVPA (CG)	BHLM 4: cfDNA AUCi	1.574	1.053	–0.492	**0.853**	**1.588**	**2.294**	3.632	1.001	3600
MVPA (IG)	BHLM 4: cfDNA AUCi	0.649	0.889	–1.102	**0.053**	**0.658**	**1.251**	2.356	1.001	7500

MCMC, Markov chain Monte Carlo; BLHM, Bayesian hierarchical-linear model; SB, sedentary behavior; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity; CG, control group; cfDNA, circulating cell-free deoxyribonucleic acid; IG, intervention group; PR, peak reactivity; AUCi, area under the curve with respect to increase; SD, standard deviation; CI, confidence interval; Rhat, potential scale reduction factor; n.eff, effective sample size.

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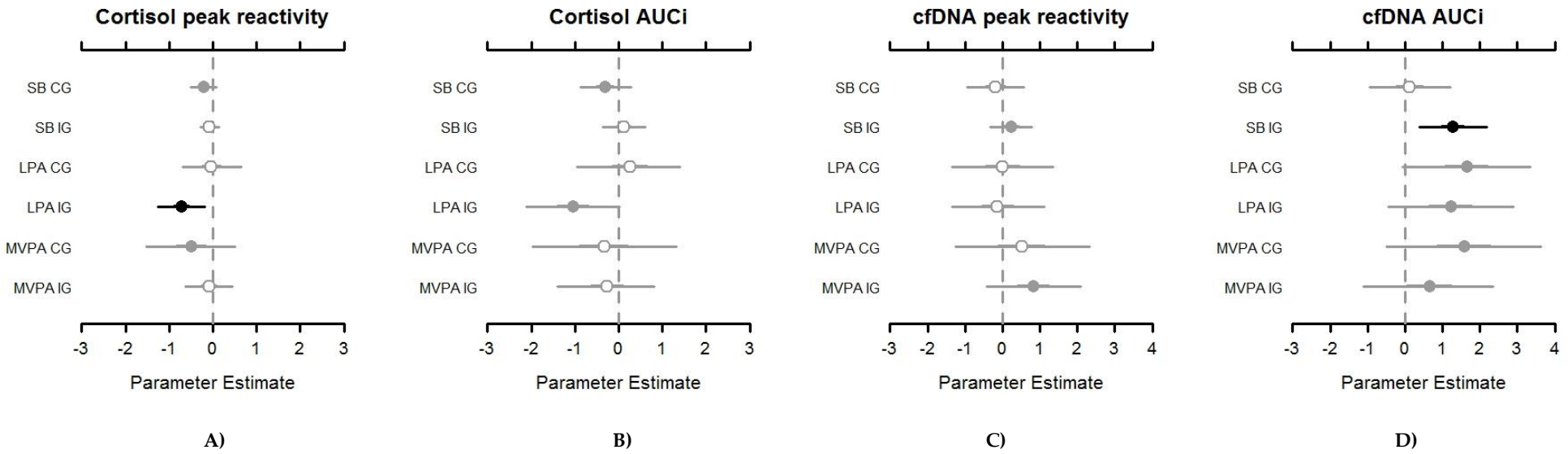

Figure 2. The highest probability density for associations among the cortisol peak reactivity (panel A)), cortisol area under the curve with respect to increase (AUCi) (panel B)), circulating cell-free deoxyribonucleic acid (cfDNA) peak reactivity (panel C)), and cfDNA AUCi (panel D)), respectively, and sedentary behavior (SB), light physical activity (LPA), and moderate-to-vigorous physical activity (MVPA), segregated by groups (CG, control group; IG, intervention group). Shading, whether 50% of confidence interval (CI; gray with open circle), 95% CI (gray with closed circle), or neither (black) do overlap 0. Black dots indicate of a strong association between the dependent and indepentend variable. For example, cortisol peak reactivity and LPA in the IG—the adverse effect of the relative amounts of LPA on the cortisol peak reactivity applies to, at least, 95% of children in the posterior distribution.

intensities are not directly comparable. Thus, it could be argued that the so-called "green effect" [8, 44] in the forest (positive effects of the NGE on humans' psychological wellbeing) supports the lowering physiological effect of relatively high LPA levels of cortisol to some extent. Perhaps, this supportive effect could be missing during the regular indoor teaching because of the buildingenvironment. The association between SB and cortsiol is more likely for the CG but not for the IG. Of note, uncomfortable sitting situations in the forest could result in psychological stress in terms of discomfort or inability to concentrate, and, therefore, potentially be attributed to this missing association. A validation study by Khoury et al. [45] reported that the PR and AUCi indices exhibit similar results regarding the cortisol increase/decrease. In this study, most associations of SB/LPA/MVPA in cortisol PR/cortisol AUCI, respectively, exhibited similar tendencies; only the tendency for a negative association of MVPA in the CG was not present for the AUCi index. Thus, we assume that our cortisol dataset based on the measurement procedure with three time points (08:30 AM, 10:30 AM, and 12:30 PM) is not entirely comparable with the time points used previously [45].

Some studies have reported that cfDNA levels already increase with moderate PA below the level of the aerobic–anaerobic transition [26, 28]. The results of the cfDNA AUCi posterior distributions suggest that such a association is also more likely in both teaching settings. However, the relative amount of SB in the IG also exhibits a strong positive association with students' cfDNA values, which, perhaps, cannot be easily explained on a theoretical or empirical basis. Also, the deviance values in both cfDNA PR/AUCi analyses are rather high compared with the respective cortisol values (cf. Tables A1–A4), which is an indication that the cfDNA MCMCs present a worse convergence compared with the cortisol MCMCs. Regarding the cfDNA, the log-likelihood is lower, and the data deviate more substantially from the models assumptions compared with cortisol. Thus, a strong positive association of SB in students' cfDNA could be likely attributed to an overestimation in the model. Regarding cfDNA results, similar tendencies have been observed between the applied PR and AUCi indices for SB and MVPA in the IG. Both indices, PR and AUCi, seem to be stable for cortisol, whereas the results for the cfDNA are more diverse regarding the PR and AUCi; this could be potentially explained by the factor "time point". As one's cortisol secretion follows a time-dependent diurnal rhythm (with expected high values in the morning and a steady decline from the noon to the evening), both validated indices account for the variation of cortisol over the period of the school day. Regarding the cfDNA, the AUCi index seems to better account for the less time-dependent and more PA-related secretion, which could be illustrated with nearly two times as much deviance for the cfDNA PR compared with the AUCi. In general, our analysis with the applied PR and AUCi indices was optimized for cortisol with its time-dependent diurnal rhythm and is therefore more appropiate to be used for cortisol compared with cfDNA.

Owing to the underlying pedagogical concept in the forest [46], students might have more breaks between phases of SB, LPA, and MVPA. Recent research has reported about positive health effects of breaks during extended periods of SB [47-50]. The possible relevance of the number of interruptions is an interesting phenomenon to be evaluated in the future research concerning students' SB and PA in school. In this study, the described differences in the pair-wise log-ratios for SB/LPA/MVPA between the CG and IG could be interpreted as a higher variability in the relative amounts of SB, LPA, and MVPA in the IG compared to the CG. Thus, it could be hypothesized that students in the forest have more freedom to choose whether they want to sit, walk, or run. During the normal indoor teaching, students frequently have to sit still for the entire 45 min in each lesson. Perhaps, the hypothesized freedom of choice could result in better physiological reactions within students' adaptive systems; this assumption is in concordance with the often proposed argument that several students cannot handle long periods of SB, as their inherent need is to be physically active. Nevertheless, in future large-scale, prospective studies, aspects of the sunlight exposure [51-52] and further possible confounder should be considered . Based on this exploratory research, more clinically controlled studies are warranted to elucidate children's cfDNA values in relation to SB and PA.

For the educational practice, however, the most important finding is that relative long time spent in LPA in an outdoor teaching setting is strongly associated with a decline of cortisol levels.

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3.2. Limitations

This study has an exploratory character that is especially based on the relatively low number of available participants along with the mentioned the higher number of uncertainties in children's cortisol and cfDNA levels in relation to their SB and PA in indoor and outdoor teaching. The chosen real-world scenario bears uncontrollable confounders. In concordance with our previous analysis [8], three measurement points over 1 school-year provided only limited insight into the complex structure of regular compulsory outdoor lessons, students' levels of measured biological stress parameters, and the respective associations with their SB and PA. However, conducting more measurement days was not feasible for logistical and school organizational reasons. In addition, several acceleration sensors fell off because of warm weather conditions during the study time point summer, which resulted in the loss of PA data. Furthermore, the allocation of students to the CG and IG was performed per the school policies and parents' choice. Thus, students could not be randomly allocated to a group by the experimenter, possibly implying certain bias. Hence, the overall small number of participants in this pilot study must be considered, and extensive, prospective studies are warranted to investigate further the tendencies explained in this study.

4. Materials and Methods

4.1 Study Design and Intervention

This exploratory analysis is part of the research project "One year in the forest–the influence of regular outdoor lessons in a natural environment on biological indicators of stress resilience." The research in the NGE comprised a great complexity concerning measurement procedures and confounding factors. Thus, in this study, we applied a mixed-methods approach in a prospective, longitudinal quasi-experimental design. In addition, functional magnetic resonance imaging, saliva cortisol and saliva cfDNA, three-axis accelerometry, and constructs of the Self-Determination Theory were used as described by Dettweiler et al. [8].

This intervention study was conducted at a secondary school in Heidelberg, Germany. Since the school-year 2013–2014, a group of fifth-grade students were taught one compulsory school day per week for the entire schoolyear in a nearby forest. The pedagogical concept of the forest teaching setting was inspired by the Scandinavian *udeskole/uteskole* approach as well as *outdoor education* from New Zealand (see [53-55] for further details). Thus, teachers intended to facilitate student-centered, hands-on, and experimental learning situations in close connection to the NGE. In addition, this change of space within the physical setting of the "classroom" implied different opportunities for problem-solving, co-operation, experimentation, and to be physically active on pupils' free choice during the lessons. Furthermore, pupils undertook regular walks to reach specific places in the forest. Of note, the contents of the lessons in the forest setting were highly connected to the formal school curriculum and were taught in cross-disciplinary units on the forest days, including a certain variance concerning the practical relevance and season. Moreover, subject-by-subject teaching was applied on standard school days for both the IG and the CG based on traditional indoor teaching concepts (refer Dettweiler et al. [8] for further information regarding timetables and von Au [46] for the pedagogical concept).

4.2. Participants and Data Collection

We enrolled participants from fifth and sixth grades from the school-year 2014–2015. In this school-year, three fifth-grade classes had forest teaching, and only one fifth-grade class had regular indoor teaching. Owing to this administrative decision of the school, we could not enroll the same number of fifth-grade students in the IG and CG. Thus, we enrolled students from a sixth-grade regular indoor teaching class into the CG; these students did not participate in the forest teaching setting during their fifth-grade school-year 2013–2014. Overall, we enrolled 48 students in this study (IG, 37; CG, 11). As some students were absent during the school-year, we could not collect datasets from all 48 students at all time points in fall, spring, and summer. Furthermore, not all saliva samples

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provided adequate material for analysis, and accidentally acceleration sensors got lost. Of note, descriptive and enrollment data for participants is presented elsewhere [8].

We collected both samples for saliva cfDNA and cortisol using Salivette®/Cortisol- Salivette® collection tubes (Sarstedt, Nümbrecht, Germany). Saliva cfDNA levels were evaluated using undiluted saliva according to the protocol described elsewhere [56]. After centrifuging at 1.600 × *g* for 2 min (room temperature), the supernatant was transferred into a new collection tube and frozen at –20°C before measurement. In addition, salivary samples for cortisol quantification were frozen at –20°C immediately after the arrival at the Biopsychology Laboratory, Technical University Dresden, and cortisol levels were determined using a commercially available luminescence immunoassay (IBL, Hamburg, Germany). Based on the validation study by Khoury et al. [45], we applied the summary indices AUCi and the PR. (For further details regarding the calculation and application of the summary indices, refer [45, 57-58] and Supplementary Material [section Material]).

We determined both SB and PA of the IG and CG using triaxial Axivity AX3 acceleration sensors (Axivity Ltd., Newcastle upon Tyne, UK). One sensor was attached to each child's back above the upper point of the posterior iliac crest, with the aid of a medical tape [43, 59]. All children were instructed not to re-attach the sensor to their skin once it fell off. All sensors were initialized at 100 Hz and ±8G bandwidth. In addition, we converted the raw vector magnitude acceleration data to ActiLife file format by an in-house software developed by the University of Southern Denmark. Children's PA levels were analyzed using ActiLife v.6.11. 4 (ActiGraph, Pensacola, FL). In addition, cut-off points reported by Romanzini et al. [60] were used to distinguish SB, LPA, and MVPA; these cut-off points have been proven to exhibit a good validity among children and adolescents to identify patterns of SB, LPA, and MVPA. However, the validity and comparability of acceleration sensors, as well as applied cut-off points, have been controversially discussed. Therefore, certain differences have to be considered when comparing studies on SB and PA, especially effects of varying epoch lengths, wear time algorithms, and activity cut-points [61-63].

4.3. Statistical Analyses

In studies on PA and health, one specific behavior is often analyzed independently from other behaviors. A recent study focused on this issue and reported that human behavior during a finite time of the day needs to be recognized as a composition that accumulates to 100% of that time. Thus, the components (e.g., sleep, SB, light PA [LPA], moderate-to-vigorous PA [MVPA]) are perfectly codependent, resulting in multicollinearity issues when applying standard multilinear regression techniques. Hence, the compositional data analysis (CoDA) [64] approach that considers all parts of the composition is recommended to provide reliable evidence on human behaviors related to health [65-66].

For data transformation, descriptive analyses, and visualization of the CoDA approach we used the software packages Compositions [67], ggplot2 [68], and ggtern [69]. To set up, document and run the hierarchical models, to evaluate associations between students' cortisol and cfDNA levels, respectively, and their relative time spent in SB, LPA and MVPA, we used ggthemes [70], jagsUI [71], rjags [72], and R2jags [73] in R 3.4.1 (2017-06-30) [74].

The usual way to fit regression models with compositional covariates is to apply isometric log-ratio (ilr) or centered log-ratio (clr) transformations on raw values, which is justified as the parts of composition perfectly correlate and standard regression techniques result in multicollinearity problems. However, the use of ilr or clr transformations poses problems with the interpretation, as the meaning of posterior parameter values remains unclear, especially in hierarchical models. Thus, in the given analysis, a Bayesian ridge regression version suggested by Parnell [75], which accepts raw compositions, was implemented and the raw composition values were transformed into a matrix using a common prior distribution function.

Further details regarding the data analysis are described in the Supplementary Material (section Methods).

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4.3.1. BHLM

The likelihood for the applied model reads

$$Y_i \sim N\left(\alpha_{\mathrm{id}_i} + \beta_{\mathrm{cmp}_i}\left(grp_i \times x_{[1:3]i}\right) + \beta_{\mathrm{ssn}_i} x_{4_i} + \beta_{\mathrm{gdr}_i} x_{5_i} + \beta_{t_i} x_{6_i}, \sigma_y^2\right), for\ i = \ 1, \ldots n$$

where

$$x_{[1:3]i} = \begin{pmatrix} SB_1 & LPA_1 & MVPA_1 \\ SB_2 & LPA_2 & MVPA_2 \\ \cdots & \cdots & \cdots \\ \cdots & \cdots & \cdots \\ SB_n & LPA_n & MVPA_n \end{pmatrix}$$

denotes the matrix of the composition of the three activity behaviors, and Table 4 presents the prior distributions of parameters in the cortisol and cfDNA models, respectively.

Table 4. The prior distribution of parameters for Bayesian hierarchical linear models

Cortisol	cfDNA
$\alpha_{id_j} \sim N(0, \sigma_\alpha^2)$	$\alpha_{id_j} \sim N(0, \sigma_\alpha^2)$
$\beta_{cmp_j} \sim N(\mu_\alpha, 1)$	$\beta_{cmp_j} \sim N(\mu_\alpha, 1)$
$\mu_\alpha \sim N\ (0, 5)$	$\mu_\alpha \sim N\ (0, 1^{-6})$
$\beta_{t_j} \sim N(0, 5)$	$\beta_{t_j} \sim N(0, 1^{-6})$
$\beta_{gdr_j} \sim N(0, 5)$	$\beta_{gdr_j} \sim N(0, 1^{-6})$
$\beta_{ssn_j} \sim N(0, 5)$	$\beta_{ssn_j} \sim N(0, 1^{-6})$
$\sigma_y^2 \sim \mathcal{HC}(0, 5)$	$\sigma_y^2 \sim \mathcal{HC}(0, 25)$
$\sigma_\alpha^2 \sim \mathcal{HC}(0, 5)$	$\sigma_\alpha^2 \sim \mathcal{HC}(0, 25)$

id, identification of participants; cmp, composition; t, time point (midmorning, noon); gdr, gender (female; male); ssn, season (fall; spring; summer).

Furthermore, we applied a different set of priors for the respective cortisol and cfDNA models, which is justified to (a) address the well-established high-variance cortisol displays (within subjects over the course of the day with higher variance later in the day, within subjects at different seasons, and between subjects and gender) and (b) as to the best of our knowledge nothing is known about children's cfDNA levels in the saliva with respect to the daytime, season, gender, SB, and PA. In this study, we allowed random intercepts (α) for each id, and put a hyper prior to α centered to zero (i.e., inform the prior from the data). In addition, we centered βcmp on $\mu\alpha$ to tie the slope parameter βcmp to the random intercepts (equivalent to nesting ids in the groups); this is called "alternative hierarchical centering" and is an elegant way to borrow strength (i.e., statistical power) from an

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individual intercept and group. Putting this prior information on the composition dissolves the problem of collinearity, which is typically addressed in ilr- or clr-transformations, which has been described as an alternative to ilr- or clr-transformation by [64], however without changing the scale of the output. Thus, the estimates could be interpreted straightforwardly. Finally, other priors were set to be normally distributed parameters around zero, with vaguely informed standard deviation for cortisol and super-vague informed standard deviation for cfDNA. Hence, the cfDNA model should be considered as a strictly provisional "reference model" [76].

In our analysis, we used log-transformed cortisol and cfDNA measures because of skewness and kurtosis (cf. Table S1). Furthermore, the Markov chains were set to 50,000 iterations, a burn-in phase of 25,000, and a thinning-rate of 10.

Supplementary Materials: Supplementary Materials S 1 Results and S 2 Methods can be found at www.mdpi.com/link.

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Author Contributions: P.K., P.S., and U.D. conceived and designed the study; C.B. collected the data; P.S., S.S. and E.N. developed tools to prepare the cfDNA-salvia probes which were statistically analyzed by C.B. and U.D.; C.B. wrote the paper with substantial contributions from all other authors. All authors proved the final version of the manuscript.

Conflicts of Interest: All authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results. The study has been approved by the internal review board of the University of Heidelberg. Written consent has been obtained. Data are stored according to international ethical and legal guidelines.

Abbreviations

AUCg	Area under the curve with respect to ground
AUCi	Area under the curve with respect to increase
BHLM	Bayesian hierarchical linear model
CG	Control group
CI	Confidence interval
cfDNA	Circulating cell-free deoxyribonucleic acid
CoDA	Compositional data analysis
DIC	Deviance information criterion
EOtC	Education outside the classroom
IG	Intervention group
LPA	Light physical activity
MCMC	Markov chain Monte Carlo
MVPA	Moderate-to-vigorous physical activity
NGE	Natural green environments
PA	Physical activity
PR	Peak reactivity
SB	Sedentary behavior
SD	Standard deviation

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Appendix A

Table A1. The MCMC output for the posterior probability for BHLM 1: sedentary behavior (SB) and physical activity (PA) in the cortisol peak reactivity; covariates group, time point, gender, and season. Values with no overlap on 0 for lower and upper CI are bold.

	Mean	SD	CI 2.5%	CI 25%	CI 50%	CI 75%	CI 97.5%	Rhat	n.eff
SB (CG)	–0.215	0.152	–0.512	**–0.318**	**–0.213**	**–0.112**	0.085	1.001	7500
SB (IG)	–0.071	0.112	–0.289	–0.144	–0.070	0.005	0.147	1.001	5600
LPA (CG)	–0.032	0.339	–0.699	–0.257	–0.032	0.193	0.635	1.001	7500
LPA (IG)	–0.728	0.271	**–1.268**	**–0.908**	**–0.726**	**–0.551**	**–0.190**	1.001	7500
MVPA (CG)	–0.499	0.524	–1.517	**–0.860**	**–0.501**	**–0.141**	0.527	1.001	5900
MVPA (IG)	–0.085	0.276	–0.623	–0.270	–0.087	0.098	0.461	1.002	2200
Season	0.007	0.023	–0.039	–0.008	0.007	0.023	0.052	1.001	7500
Gender male	0.028	0.041	–0.054	0.000	0.028	0.056	0.110	1.002	2100
Time point noon	0.079	0.041	0.000	**0.052**	**0.079**	**0.107**	0.160	1.001	7500
Sigma	0.265	0.013	0.241	0.256	0.264	0.273	0.291	1.001	6200
Deviance	39.402	4.902	31.117	35.975	38.908	42.325	50.507	1.001	7500

MCMC, Markov chain Monte Carlo; BLHM, Bayesian hierarchical-linear model; SB, sedentary behavior; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity; CG, control group; IG, intervention group; Time point (midmorning, noon); Gender (female, male); Season (fall, spring, or summer); SD, standard deviation; CI, confidence interval; Rhat, potential scale reduction factor; n.eff, effective sample size.

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Table A2. The MCMC output for the posterior probability for BHLM 2: sedentary behavior (SB) and physical activity (PA) in the cortisol AUCi; covariates group, gender, and season. Values with no overlap on 0 for lower and upper CI are bold.

	Mean	**SD**	**CI 2.5%**	**CI 25%**	**CI 50%**	**CI 75%**	**CI 97.5%**	**Rhat**	**n.eff**
SB (CG)	–0.293	0.298	–0.880	**–0.494**	**–0.291**	**–0.095**	0.297	1.001	7100
SB (IG)	0.118	0.245	–0.359	–0.047	0.114	0.282	0.608	1.001	7500
LPA (CG)	0.246	0.596	–0.919	–0.156	0.250	0.643	1.404	1.001	7500
LPA (IG)	–1.027	0.550	–2.112	**–1.403**	**–1.030**	**–0.661**	0.062	1.001	6700
MVPA (CG)	–0.316	0.826	–1.947	–0.880	–0.316	0.235	1.321	1.002	1900
MVPA (IG)	–0.258	0.581	–1.400	–0.649	–0.267	0.136	0.895	1.002	2300
Season	0.014	0.037	–0.057	–0.011	0.014	0.038	0.085	1.001	4300
Gender male	0.046	0.098	–0.147	–0.020	0.047	0.111	0.239	1.001	6700
Sigma	0.288	0.027	0.241	0.270	0.286	0.305	0.347	1.002	2800
Deviance	37.777	14.434	12.005	27.371	36.864	47.052	69.210	1.001	3600

MCMC, Markov chain Monte Carlo; BLHM, Bayesian hierarchical-linear model; SB, sedentary behavior; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity; CG, control group; IG, intervention group; Gender (female, male); Season (fall, spring, or summer); SD, standard deviation; CI, confidence interval; Rhat, potential scale reduction factor; n.eff, effective sample size.

Table A3. The MCMC output for the posterior probability for BHLM 3: sedentary behavior (SB) and physical activity (PA) on circulating cell-free deoxyribonucleic acid (cfDNA) peak reactivity; covariates group, time point, gender, and season. Values with no overlap on 0 for lower and upper CI are bold.

	Mean	**SD**	**CI 2.5%**	**CI 25%**	**CI 50%**	**CI 75%**	**CI 97.5%**	**Rhat**	**n.eff**
SB (CG)	–0.181	0.391	–0.955	–0.444	–0.179	0.087	0.576	1.001	7500
SB (IG)	0.249	0.288	–0.319	**0.055**	**0.249**	**0.445**	0.796	1.001	7500
LPA (CG)	0.009	0.689	–1.356	–0.448	0.007	0.480	1.346	1.001	4300
LPA (IG)	–0.126	0.631	–1.361	–0.549	–0.129	0.299	1.109	1.001	4800
MVPA (CG)	0.512	0.917	–1.262	–0.107	0.513	1.131	2.332	1.001	7500
MVPA (IG)	0.839	0.636	–0.418	**0.416**	**0.839**	**1.269**	2.088	1.001	7500
Season	–0.108	0.062	–0.231	**–0.150**	**–0.109**	**–0.066**	0.015	1.001	6400
Gender male	–0.147	0.110	–0.362	**–0.220**	**–0.147**	**–0.074**	0.072	1.001	7500
Time point noon	0.253	0.108	0.038	0.181	0.254	0.327	0.461	1.001	7500
Sigma	0.675	0.035	0.611	0.651	0.674	0.698	0.748	1.001	7300
Deviance	411.222	4.392	404.037	408.108	410.696	413.810	421.139	1.001	7500

MCMC, Markov chain Monte Carlo; BLHM, Bayesian hierarchical-linear model; SB, sedentary behavior; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity; CG, control group; IG, intervention group; Time point (midmorning, noon); Gender (female, male); Season (fall, spring, or summer); SD, standard deviation; CI, confidence interval; Rhat, potential scale reduction factor; n.eff, effective sample size.

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Table A4. The MCMC output for the posterior probability for BHLM 4: sedentary behavior (SB) and physical activity (PA) on circulating cell-free deoxyribonucleic acid (cfDNA) AUCi; covariates group, gender, and season. Values with no overlap on 0 for lower and upper CI are bold.

	Mean	**SD**	**CI 2.5%**	**CI 25%**	**CI 50%**	**CI 75%**	**CI 97.5%**	**Rhat**	**n.eff**
SB (CG)	0.125	0.555	–0.955	–0.242	0.124	0.498	1.208	1.001	3800
SB (IG)	1.285	0.464	**0.390**	**0.970**	**1.286**	**1.595**	**2.191**	1.001	7500
LPA (CG)	1.643	0.877	–0.072	**1.058**	**1.652**	**2.232**	3.348	1.001	4300
LPA (IG)	1.231	0.858	–0.455	**0.647**	**1.227**	**1.804**	2.899	1.001	5300
MVPA (CG)	1.574	1.053	–0.492	**0.853**	**1.588**	**2.294**	3.632	1.001	3600
MVPA (IG)	0.649	0.889	–1.102	**0.053**	**0.658**	**1.251**	2.356	1.001	7500
Season	–0.264	0.101	**–0.461**	**–0.332**	**–0.262**	**–0.196**	**–0.067**	1.001	7500
Gender male	–0.283	0.186	–0.651	**–0.406**	**–0.285**	**–0.157**	0.083	1.001	7500
Sigma	0.759	0.061	0.647	0.717	0.757	0.798	0.888	1.001	4100
Deviance	221.887	7.045	205.152	218.423	222.668	226.326	234.212	1.002	1200

MCMC, Markov chain Monte Carlo; BLHM, Bayesian hierarchical-linear model; SB, sedentary behavior; LPA, light physical activity; MVPA, moderate-to-vigorous physical activity; CG, control group; IG, intervention group; Gender (female, male); Season (fall, spring, or summer); SD, standard deviation; CI, confidence interval; Rhat, potential scale reduction factor; n.eff, effective sample size.

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