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PSYCHIATRY AND PRECLINICAL PSYCHIATRIC STUDIES - ORIGINAL ARTICLE

Investigating the development of the autonomic nervous system in infancy through pupillometry

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Abstract

We aim to investigate early developmental trajectories of the autonomic nervous system (ANS) as indexed by the pupillary light reflex (PLR) in infants with (i.e. preterm birth, feeding difficulties, or siblings of children with autism spectrum disorder) and without (controls) increased likelihood for atypical ANS development. We used eye-tracking to capture the PLR in 216 infants in a longitudinal follow-up study spanning 5 to 24 months of age, and linear mixed models to investigate effects of age and group on three PLR parameters: baseline pupil diameter, latency to constriction and relative constriction amplitude. An increase with age was found in baseline pupil diameter ($F(3,273.21) = 13.15$, $p < 0.001$, $\eta_p^2 = 0.13$), latency to constriction ($F(3,326.41) = 3.84$, $p = 0.010$, $\eta_p^2 = 0.03$) and relative constriction amplitude($F(3,282.53) = 3.70$, $p = 0.012$, $\eta_p^2 = 0.04$). Group differences were found for baseline pupil diameter ($F(3,235.91) = 9.40$, $p < 0.001$, $\eta_p^2 = 0.11$), with larger diameter in preterms and siblings than in controls, and for latency to constriction ($F(3,237.10) = 3.48$, $p = 0.017$, $\eta_p^2 = 0.04$), with preterms having a longer latency than controls. The results align with previous evidence, with development over time that could be explained by ANS maturation. To better understand the cause of the group differences, further research in a larger sample is necessary, combining pupillometry with other measures to further validate its value.

Keywords Pupillometry · Infant development · Autonomic nervous system · Autism spectrum disorder · Preterm birth

Introduction

The development of the autonomic nervous system (ANS) in infancy has gained interest over the past few years. The ANS is vital in regulating the body's physiology when coping with stress, via the complementary functioning of the sympathetic (SNS) and parasympathetic nervous system (PNS) (Wehrwein et al. 2016). Cerritelli et al. (2021) recently reviewed literature on the ANS in fetal, perinatal and postnatal life to determine 'critical windows' in development. Here, we focus on three groups of children who may be particularly prone for atypical ANS development.

First, *prematurely born children* often show an atypical ANS, which may be modulated by gestational age and comorbidities, and should be considered by clinicians (Cerritelli et al. 2021; Fyfe et al. 2015; Schlatterer et al. 2022). Neuropathological evidence suggests that the amount of myelinated fibres of the vagus nerve, an important part of the PNS, in preterm infants born after 24 weeks gestational age increases linearly with age until about 40 weeks postconceptional age, whereas term infants at birth already

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have a similar amount of myelinated fibres as adolescents do (Sachis et al. 1982), with possibly an additional slow increase in the first 3 months of their life (Pereyra et al. 1992). In term infants, active myelination seems to be present in the first 9 months of life, with an increase of the amount of myelin within myelinated fibres (Pereyra et al. 1992). In preterm infants, long-term fibre tract alterations remain present even at the ages of 7 and 13 (Kelly et al. 2020).

Second, atypical ANS functioning has been demonstrated in *children with autism spectrum disorder* (Arora et al. 2021; Hutt et al 1964). In this population, evidence is equivocal, with arguments both for hyperarousal (Hutt et al. 1964) and hypo-arousal (Rogers and Ozonoff 2005) at different ages (Arora et al. 2021). The vagal nerve is the largest nerve in the parasympathetic nervus system (Breit et al. 2018). Within the context of the polyvagal theory, Porges (2007) suggests that underlying neurobiological state regulation strategies could be obstructing social behaviors through a vagal brake. Social abilities are optimal when arousal is at a normal level, but when arousal increases because danger in the environment is detected, social behaviors are compromised. This could be the case in children with autism spectrum disorder (ASD), where social problems are a core symptom. Infants with older siblings with ASD, and therefore an increased likelihood of developing ASD themselves, may also have an atypical ANS development.

Third, *children with eating or feeding problems* also often display an atypical ANS (Porges 2007; Porges and Lipsitt 1993; Quigley et al 2017). The gustatory-vagal hypothesis (Porges and Lipsitt 1993) postulates that nutritive sucking in an experimental settings can regulate the psychophysiological state of infants. The parasympathetic vagal nerve is of utmost important in the brain-gut axis, mostly transferring information from the intestines to the brain (afferent function) and for a small part influencing digestion (efferent function). It is seen as a link between the central and the enteric nervous system (Breit et al. 2018). Cardiac vagal tone parallels sucking frequency, associated with gustatory stimulation, and this was recently shown to extend to infants during breastfeeding (Quigley et al. 2017). The particular diet of an infant may also impact upon the stability of the general vagal tone (Pivik et al. 2015), which makes infants with eating/feeding difficulties (e.g. selective eating) an additional interesting group to investigate.

While most current evidence on ANS development in infants and young children is based on measurements of heart-rate variability (HRV) (Javorka et al. 2017; Lavanga et al. 2021), pupillometry has been proposed as a novel and non-invasive method for studying the ANS (Hall et al 2018).

Pupillometry is the measurement of the changes in pupil size over time in response to a stimulus. The pupillary light reflex (PLR) is characterized by a decrease of the pupil diameter in response to increased luminance detection on the retina, followed by an increase to baseline. This reflex is mediated by the ANS, with the pupil being both parasympathetically and sympathetically innervated, as described by Lowenstein and Loewenfeld (1950), and more recently by amongst others (Hall et al. 2018; Wehrwein et al. 2016). Light passes through the pupil, falls on the retina and activates a signal that is transferred through the optic nerve, onwards to the pretectal nuclei. Then, the nuclei of Edinger Westphal are activated, with the signal continuing on to the preganglionic parasympathetic fibers along the oculomotor nerve, synapsing in the ciliary ganglion and ending in the sphincter muscle of the iris, resulting in miosis, a contraction of the pupil. Sympathetic innervation of the pupil dilator muscle results in mydriasis. The locus coeruleus (LC) is part of the ANS and is an important nucleus in the brainstem, releasing norepinephrine (NE) and is thus essential in regulating arousal. The LC–NE system influences the parasympathetic Edinger–Westphal nucleus (Lynch 2018; Samuels and Szabadi 2008) and the sympathetic superior cervical ganglion which connects to the dilator muscle (Nobukawa et al. 2021) and consequently influences the PLR. All these structures and their projections are resulting in the pupillary light response, reflecting a balance of both parasympathetic and sympathetic innervation. When the PLR is altered, this can be caused by alterations on all these levels. The LC–NE system has been studied in neurodiverse populations, implying a possible underlying role of aberrant development of this system in neurodevelopmental conditions such as ASD and ADHD (Bast et al. 2021).

Three main parameters are reported in most studies studying the PLR: baseline pupil diameter, latency to constriction and relative constriction amplitude, see Fig. 1.

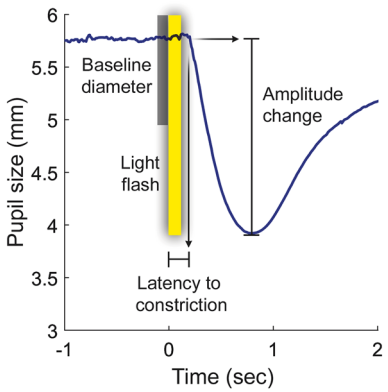

Fig. 1 Pupil size changes over time in response to light stimulus

In typically developing children, a positive correlation was found between age and the maximum and minimum pupil diameter (Brown et al. 2015; Shah et al. 2020). Evidence on relative constriction amplitude is mixed, with some authors finding a positive correlation with age (Kercher et al. 2020; Shah et al. 2020) and others stating the opposite (Winston et al. 2020). Baseline pupil diameter generally increases with age (Kercher et al. 2020; Winston et al. 2020), whereas latency to constriction decreases with age (Kercher et al. 2020). Twin studies show that heritability also plays a role in baseline pupil diameter and the relative constriction amplitude (Portugal et al. 2021), and that these two parameters are related to the distinct genetic factors.

The present study aims at characterizing the development of the pupillary light reflex over the first 2 years of life in the three abovementioned groups of infants with increased likelihood of atypical ANS development, as well as infants without this increased likelihood.

Methods

Sample

The current data are collected as part of the TIARA (Tracking Infants At Risk for ASD) study, which is a collaboration between KU Leuven and Ghent University. The TIARA study is a prospective longitudinal study, where infants enrolled at the age of 5 months (or exceptionally at 10 or 14 months of age) and were followed until the age of 36 months. Data was collected between January 2018 and December 2021. A total of 216 infants were included in this experiment, after selection based on data quality, each of them participating one to four times. This resulted in 460 observations. Demographics of included participants and number of excluded infants in each group can be found in Table 1.

Approval by the ethical committee at both sites was granted. Parents gave informed consent to process and report

Table 1 Demographic characteristics of included infants at each timepoint

Group	*n*	Male		Female		Age (days)		*n* excl
		n	%	*n*	%	M	SD	
T1—5 m								
Siblings ASD	49	25	51	24	49	161	23	14
Preterms	45	22	49	23	51	168	16	16
Feeding difficulties	4	4	100	0	0	162	15	0
Controls	19	11	58	8	42	154	29	4
Total	117	62	53	55	47	163	22	34
T2—10 m								
Siblings ASD	78	43	55	35	45	312	21	10
Preterms	50	24	48	26	52	323	19	11
Feeding difficulties	9	6	67	3	33	306	10	0
Controls	16	9	56	7	44	301	25	4
Total	153	82	54	71	46	314	20	25
T3—14 m								
Siblings ASD	72	39	54	33	46	432	22	15
Preterms	43	23	53	20	47	443	25	8
Feeding difficulties	11	7	64	4	36	431	14	0
Controls	13	7	54	6	46	432	14	2
Total	139	76	55	63	45	435	23	25
T4—24 m								
Siblings ASD	29	15	52	14	48	739	36	9
Preterms	13	6	46	7	54	752	26	7
Feeding difficulties	3	2	66	1	33	801	60	0
Controls	6	3	50	3	50	766	36	1
Total	51	26	51	25	49	749	39	17

n: number of included infants with qualitative data. *n* excl: number of infants that did participate in the eye-tracking tasks, but data had to be excluded due to lack of sufficient amount of qualitative data. Siblings ASD: infants with an older sibling with a diagnosis of autism spectrum disorder. Preterms: infants born before 30 weeks gestational age. Feeding difficulties: infants with medically insufficiently explained feeding or eating difficulties

on the collected data. Data was collected at both sites. We recruited three groups of infants with the help of clinical and societal partners (regional hospitals, well-child check-ups, …): (1) siblings of children with a diagnosis of ASD (from now on referred to as 'siblings'), (2) preterms, born before 30 weeks gestational age and (3) children with medically insufficiently explained eating or feeding problems (referred to as 'feeding difficulties'), which was added from an exploratory perspective, due to little existing research in this population. Sample size was planned to at least exceed previous studies and was only restricted to how many parents were willing to join within the project time limits. In addition, in Leuven, a group of term-born (i.e. after 37 weeks of pregnancy) control infants was recruited, who did not fulfil any of the criteria of the previously listed groups (referred to as 'controls'). Exclusion criteria were epilepsy, known genetic diagnoses associated with an increased chance of developing ASD, significant medical issues that could influence brain development or participation in the study, presence of uncorrected visual or auditory problems, not growing up with at least one biological parent. Eye-tracking was performed at the ages of 5, 10, 14 and 24 months, with corrected ages for children born before 37 weeks of gestation. The goal was to repeat the experiment four times in all infants, to acquire a longitudinal dataset. Unfortunately, due to COVID-19 restrictions, some children missed several timepoints.

Measures and procedure

At both sites a Tobii eyetracker (Tobii Technology, Danderyd, Sweden) was used, in Ghent the T60 model, in Leuven the X3-120 model. The infants were seated on their parents laps while watching a screen at approximately 60 cm, in a room with controlled ambient lighting. Pupillary light reflex was tested by using the established paradigm of Nyström et al. (2015), which was presented in between other eye-tracking tasks. A maximum of twelve trials per session were collected, infants with less than four valid trials were excluded. To control our data quality, we investigated the proportion of trials that were considered valid in the included infants per age group.

Participants watched a black screen with a centrally presented attention grabber for 1666 ms, when the background changed to white for 120 ms (perceived as a light flash), before returning black for another 4000 ms. Paradigms were presented using MATLAB software R2019B (The Mathworks Inc 2019) and Psychtoolbox 3 (Brainard 1997).

Baseline pupil diameter (D_0) was defined as the mean pupil diameter in the 100 ms interval prior to the flash. Latency to constriction was defined as the duration from the start of the flash until the absolute maximum acceleration in the following 100 to 500 ms interval, as suggested by Bergamin and Kardon (2003). Latency to constriction was calculated based on the absolute minimum of the second derivative of the pupil size time series (acceleration), with the first derivative being the velocity, which resulted in the timepoint where the change of pupil size (velocity) differs the most, meaning the maximum acceleration (see Bergamin and Kardon 2003). Relative constriction amplitude was calculated in line with Fan et al. (2009), with the formula $(D_0^2 - D_m^2)/D_0^2$, after calculating the minimum diameter (D_m) in the 500–1500 ms post-flash interval.

Data processing

Preprocessing was done in TimeStudio (Nyström et al. 2016) using MATLAB software R2019b (The Mathworks Inc 2019), in accordance to Nyström et al. (2015). Gaps in the data were linearly interpolated (with a maximum of two samples) and trials where participants looked at the screen for < 90% of the 0–1000 ms post-flash time interval were excluded for baseline pupil diameter and latency to constriction analyses. In the analyses of the relative constriction amplitude, we decided at thresholding at 75% looking time, due to a longer analysis interval (0–1500 ms), which would have resulted in more data loss. Sessions yielding less than four valid trials were excluded from the analysis. A five-point second-order polynomial moving Savitzky–Golay filter was applied before resampling the signal to 300 Hz to achieve better temporal resolution (Bergamin and Kardon 2003). A centred 25-point moving average filter was applied to the 300 Hz pupil traces before further processing.

Statistical analysis

Data analysis on the extracted PLR parameters was performed in R version 4.1.2 with RStudio software version 2021.09.2 (R Core Team 2018). For each of the three PLR parameters (baseline pupil diameter, latency to constriction and relative constriction amplitude), we conducted linear mixed models (LMM) with the Afex package (Singmann et al. 2021), which is dependent on the lme4 package (Bates et al. 2015), to examine the development of the parameter over time in the four participant groups. Based on this research question, we entered group and timepoint as fixed effects, as well as the interaction between them, and subject as a random intercept to account for the nested nature of the data (repeated measures) and within-subject correlated variance. This led to the model: PLR ~ Group * Time + (1 | Subject). Estimated marginal means (EMM) will be reported as $M_{group/time}$, e.g. M_p for the EMM of the preterm group, M_{10} for the EMM at 10 months.

Prior to running the final analyses, we verified the influence of sex and the presence of retinopathy of prematurity, but neither of these factors had a significant effect and they were therefore excluded from our final model. These

analyses can be found in the supplementary file Online Resource 1. Data quality and sampling rate were also not included as covariates in the final model, but the results of the model with these two parameters are shown in Online Resource 2, along with further explanation on why we did not include these parameters in our final model. We also decided against using the other two PLR parameters as covariates to predict the third one, as this did not alter our results and was not included in our research question. We calculated p-values with the Satterthwaite's method, as well as effect sizes by converting F-values and t-values to η_p^2 via the package effectsize (Makowski et al. 2020). Post-hoc group comparisons were Bonferroni corrected and standardized estimated marginalized mean differences are reported.

The assumption of linearity was checked and confirmed for each of the models. While the assumption of normal distribution of the residuals (Shapiro–Wilk normality test), was not met for the models for baseline pupil diameter and relative constriction amplitude, we nonetheless used these mixed models, because of their established robustness against these violations (Schielzeth et al. 2020) and our relatively large sample size.

Results

Baseline pupil diameter

A LMM on the data of the baseline pupil diameter revealed significant main effects of group ($F(3,235.91) = 9.40$, $p < 0.001$, $\eta_p^2 = 0.11$), and timepoint ($F(3,273.21) = 13.15$, $p < 0.001$, $\eta_p^2 = 0.13$), but did not show any interaction effects ($F(9, 272.20) = 0.98$, $p = 0.458$, $\eta_p^2 = 0.03$). Follow-up pairwise comparisons showed significant differences between controls and preterms (estimate = − 0.685, 95% *CI* [− 1.044, − 0.326], $t(248) = -5.074$, $p < 0.0001$), as well as between controls and siblings (estimate = − 0.347, 95% *CI* [− 0.687, − 0.006], $t(244) = -2.709$, $p = 0.0434$) and preterms and siblings (estimate = 0.339, 95% CI [0.086, 0.591], $t(233) = 3.565$, $p = 0.0026$). Controls had the smallest baseline pupil diameter ($M_C = 3.47$ mm, 95% CI [3.25, 3.69]), followed by siblings of children with a diagnosis of ASD ($M_s = 3.82$ mm, 95% CI [3.70, 3.93]), children with feeding difficulties ($M_F = 3.86$ mm, 95% CI [3.51, 4.21]) and finally the preterms ($M_P = 4.16$ mm, 95% CI [4.01, 4.30]). The baseline pupil diameter increased with age, with significant differences between timepoints 5 months and 14 months (estimate = − 0.331, 95% *CI* [− 0.528, − 0.134], $t(293) = -4.470$, $p = 0.0001$), 5 months and 24 months (estimate = − 0.569, 95% *CI* [− 0.826, − 0.312], $t(279) = -5.874$, $p < 0.0001$), 10 months and 24 months (estimate = − 0.366, 95% *CI* [− 0.601, − 0.131], $t(270) = -4.139$, $p = 0.0003$) and 14 months and 24 months (estimate = − 0.238, 95% *CI* [− 0.469, − 0.007], $t(269) = -2.735$, $p = 0.0399$), as shown in Fig. 2.

Latency to constriction

A main effect of group ($F(3,237.10) = 3.48$, $p = 0.017$, $\eta_p^2 = 0.04$) and time ($F(3,326.41) = 3.84$, $p = 0.010$, $\eta_p^2 = 0.03$) was also present for the latency to constriction parameter, see Fig. 3. Preterms ($M_p = 0.340$ s, 95% *CI* [0.327, 0.354]) had a significantly longer latency to constriction than the control group ($M_c = 0.300$ s, 95% *CI* [0.278, 0.321], estimate = − 0.04, 95% *CI* [− 0.076, − 0.006], $t(267) = -3.106$, $p = 0.0126$). The latency to constriction increased with age ($M_5 = 0.299$ s 95% *CI* [0.280, 0.317], $M_{10} = 0.328$ s 95% *CI* [0.314, 0.343], $M_{14} = 0.336$ s 95% *CI* [0.321, 0.350], $M_{24} = 0.317$ 95% *CI* [0.293, 0.342]), with significant differences between 5 and 10 months (estimate = − 0.029, 95% *CI* [− 0.059, 0.001], $t(351) = -2.622$, $p = 0.0548$) and 5 and 14 months of age (estimate = − 0.037, 95% *CI* [− 0.067, − 0.007], $t(362) = -3.273$, $p = 0.0070$). There was no significant group by time interaction effect ($F(9,325.28) = 1.10$, $p = 0.361$).

Relative constriction amplitude

A LMM on this parameter only showed a significant main effect of time ($F(3,282.53) = 3.70$, $p = 0.012$, $\eta_p^2 = 0.04$), and no effect of group ($F(3,235.83) = 1.05$, $p = 0.372$, $\eta_p^2 = 0.01$) nor group by time interaction ($F(9,281.35) = 0.98$, $p = 0.460$, $\eta_p^2 = 0.03$). Similar to the latency to constriction parameter, pairwise comparisons show a significant increase of relative constriction amplitude between 5 and 10 months (estimate = − 0.034, 95% *CI* [− 0.067, − 0.002], $t(306) = -2.822$, $p = 0.0305$), and 5 and 14 months (estimate = − 0.040, 95% *CI* [− 0.073, − 0.007], $t(314) = -3.208$, $p = 0.0089$), but after this first increase, the relative constriction amplitude remains stable over time ($M_5 = 34{,}8\%$, 95% *CI* [32.5%, 37.1%]; $M_{10} = 38.3\%$, 95% *CI* [36.4%, 40.2%]; $M_{14} = 38.8\%$, 95% *CI* [36.9%, 40.7%]; $M_{24} = 37.9\%$, 95% *CI* [35.1%, 40.8%]), as can be seen in Fig. 4.

The supplementary table in Online Resource 3 shows the values of the estimated marginal means, as pictured in Figs. 2, 3 and 4.

The proportion of valid trials per age and per group can be seen in Fig. 5 for all three pupil parameters. This proportion differed significantly per age group for all three parameters: baseline pupil diameter χ^2 (3, $N = 5520$) = 22.55, $p < 0.001$ latency to constriction χ^2 (3, $N = 5520$) = 30.02, $p < 0.001$, and finally relative constriction amplitude χ^2 (3, $N = 5520$) = 28.15, $p < 0.001$ as well. When comparing data quality across groups, we find no significant

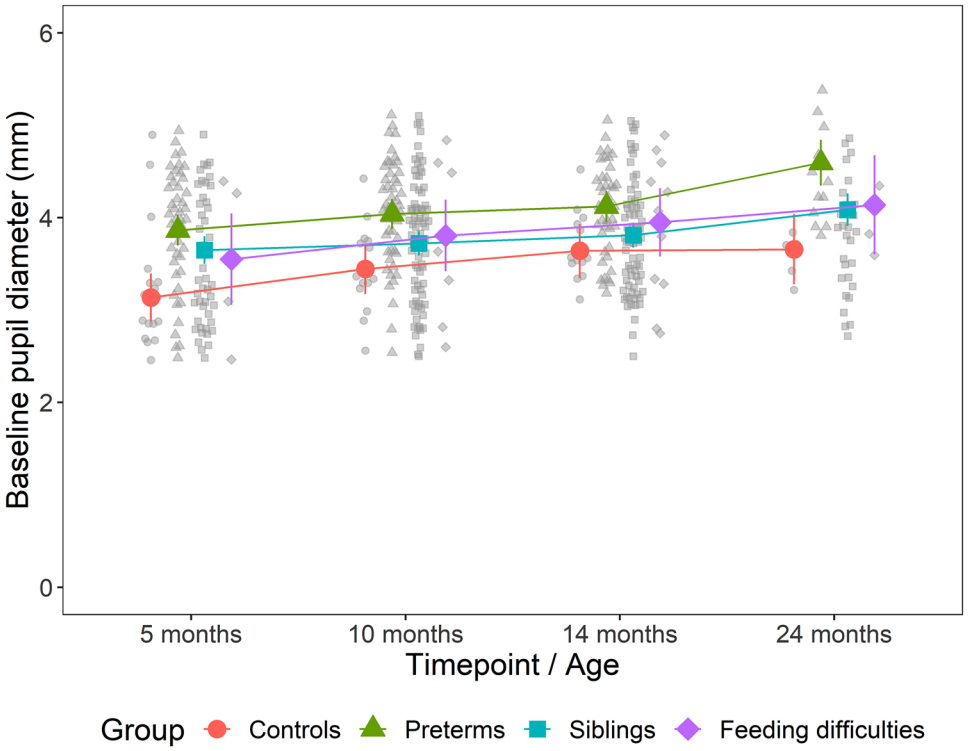

Fig. 2 Development of estimated marginal means (EMM) with standard errors of baseline pupil diameter over time for each of the participant groups. Individual data points are plotted in grey

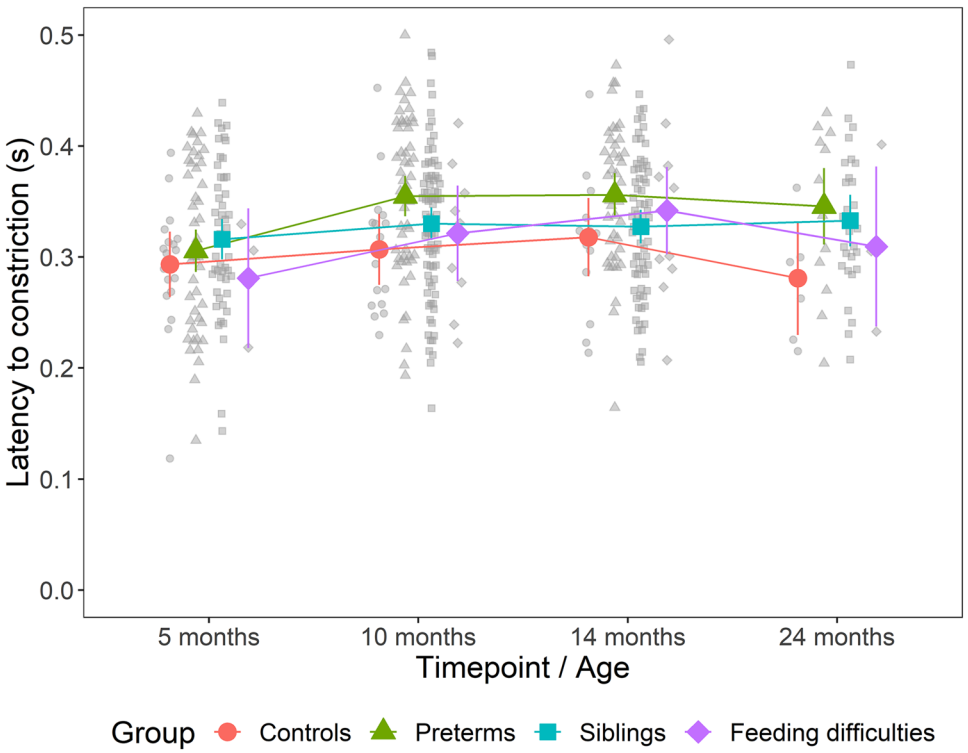

Fig. 3 Development of estimated marginal means (EMM) with standard errors of latency to constriction over time for each of the participant groups. Individual data points are plotted in grey

difference in the latency to constriction parameter (χ^2 (3, $N = 5520$) = 5.65, p = 0.130. However, significant differences across groups were found in the baseline pupil diameter (χ^2(3, $N = 5520$) = 19.98, p < 0.001) and relative constriction amplitude (χ^2 (3, $N = 5520$) = 18.56, p < 0.001) Violin

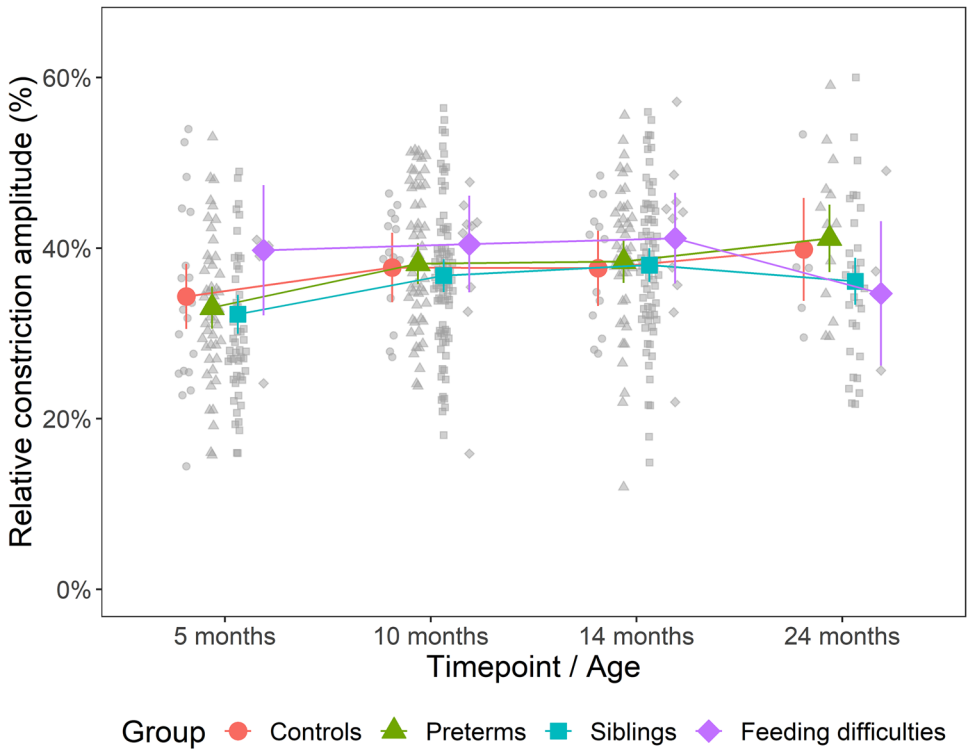

Fig. 4 Development of estimated marginal means (EMM) with standard errors of relative pupil constriction amplitude over time for each of the participant groups. Individual data points are plotted in grey

plots picturing data availability per age and per group can be found in Online Resource 4.

Discussion

This study revealed significant developmental evolution in the pupillary light reflex over the first 2 years of life in infants with and without an increased likelihood for an atypical ANS. The three parameters of interest (baseline pupil diameter, latency to constriction, relative constriction amplitude) increase with increasing age. Our findings of this maturation effect in baseline pupil diameter and relative constriction amplitude concur with previous research in typically developing term children (Brown et al. 2015; Shah et al. 2020; Winston et al. 2020), but the increase in latency to constriction was not in accordance to previous findings. Research on all these PLR parameters in typically developing infants of this age is still limited, so there is a need for future research to establish normative trajectories, to be able to pinpoint possible atypical trajectories in differently developing groups. Combining pupillometry with cardiac measures such as heart rate variability or respiratory sinus arrhythmia, which are well studied in infants (Javorka et al. 2017; Lavanga et al. 2021), could give us even more insight in the development of the ANS and the clinical and theoretical significance of PLR measures. Animal studies on the maturation of pupillary light reflex in early life and the correlation with other ANS measurements are limited but could give more insight in underlying physiological maturation.

In terms of group comparisons, we found significant group differences in these developmental trajectories, in particular for the preterm sample. In prematurely born children, the baseline pupil diameter was significantly larger than in the control group and the latency to constriction is significantly longer. Preterms often have an atypical visual development (Tremblay et al. 2014), especially of the magnocellular retinal ganglion cells (RGC). However, the pupillary light reflex is mediated by multiple pathways (Hattar et al. 2003), including also the intrinsically photosensitive RGC, which have not yet been proven to develop differently in this group. We verified whether retinopathy, a frequently co-occurring visual disorder in preterms, may have influenced our results. Yet, this was not the case, as was to be expected by previous studies (Bowl et al. 2019), but it is important to investigate the influence of gestational age in a larger cohort of infants, as we only included very and extreme preterm born children with a gestational age of less than 30 weeks. Due to the limited sample size, we were not able to investigate a possible influence of gestational age within this group, but we would recommend this for future research. According to Cerritelli et al. (2021), from 25 weeks of gestational age until birth, a critical window for parasympathetic nervous system development is present, so we would expect infants born after a shorter gestation to differ even more from a term population. As stated earlier,

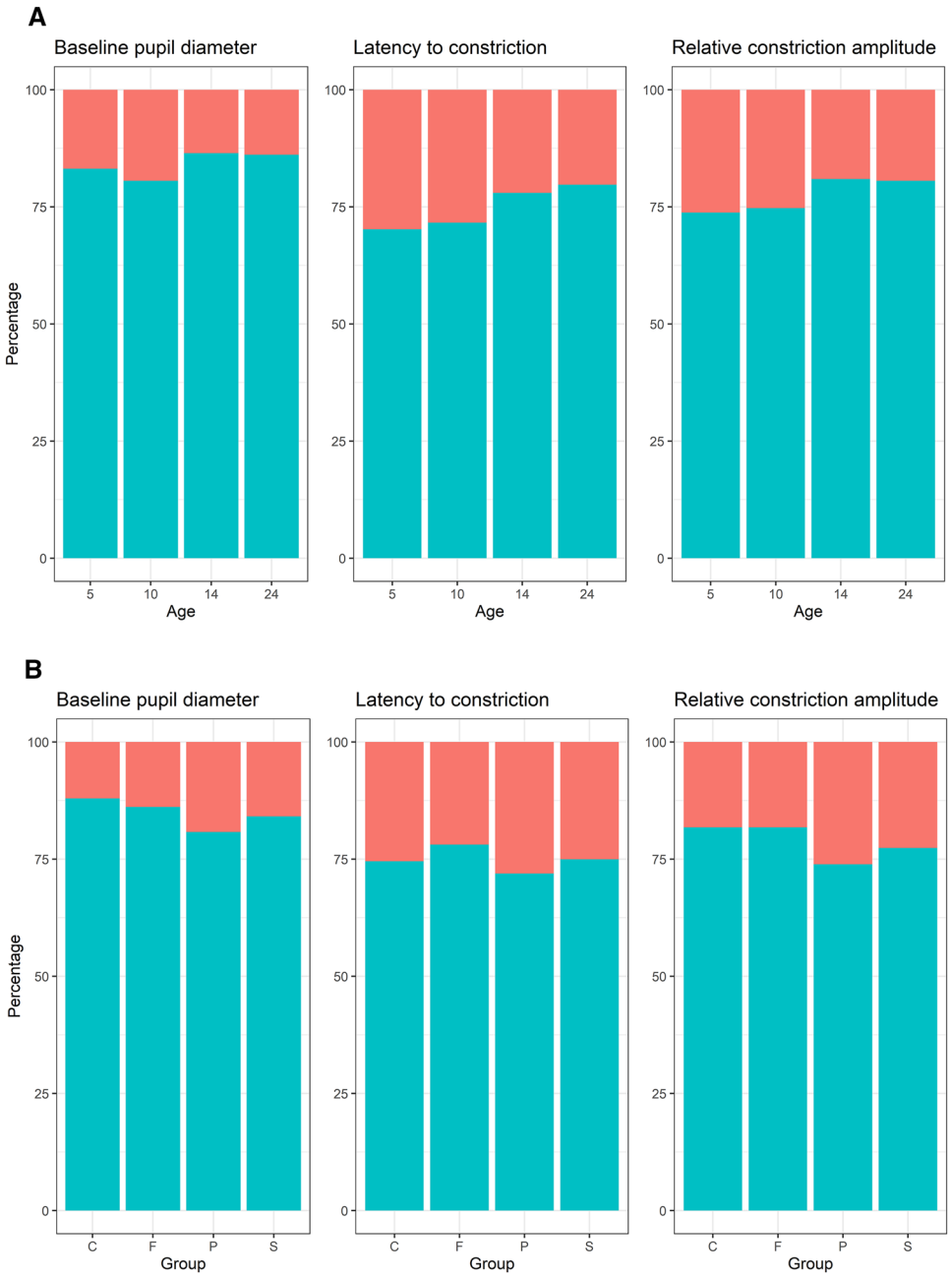

Fig. 5 Proportion of trials included or eliminated for all three PLR parameters. Green represents the proportion of valid trials, red represents the eliminated trials. Panel **A**: distribution at each timepoint/age. Panel **B**: distribution per group, *C* controls, *F* feeding difficulties, *P* preterms, *S* siblings of children with ASD

preterms are known to have an atypical developmental pattern of myelination of the vagal nerve in the first weeks and months of their life, as compared to term children (Pereyra et al. 1992; Sachis et al. 1982). Nerve myelination enhances signal transmission (Nave and Werner 2014; Tasaki 1939) and because of our current findings, we now hypothesize that vagal myelination in preterms extends to the broader PNS, including the nerves involved in the PLR. While we believe this may be an important explanatory factor for the group differences, myelination differences cannot explain the main time effect that was present, as we would then expect the latency to constriction to decrease instead of increase. When studying perinatal white matter maturation, myelination is not the only important process. Growth and pruning of the axonal fibers, connections forming between different brain areas, are all happening simultaneously in these first months of life (Dubois et al. 2014) and therefore it is hard to delineate which developmental process is reflected by our results.

Children with an older sibling with an ASD diagnosis also had a larger baseline pupil diameter than controls. Previous evidence on this finding is conflicting, with a recent meta-analysis showing no consistent difference in baseline pupil diameter between individuals with versus without ASD across all ages combined (de Vries et al.

2021). Kercher et al. (2020) studied children in the same age range as ours and also found an increased baseline pupil diameter in the group with increased likelihood of developing ASD, as well as Camero et al. (2021) who found a non-significant trend in the same direction. The study of Dinalankara et al. (2017) also shows a larger baseline pupil diameter in a group of 2 years old with an ASD diagnosis, but a decreasing baseline pupil diameter in the ASD group at older ages, and an increase in the typically developing group. However, Nyström et al. (2015, 2018) did not find differences in baseline pupil diameter.

We could not identify differences in latency to constriction or relative constriction amplitude, which is also in contrast with previous findings. Based on several previous findings, we would expect a longer latency to constriction in the ASD group after the age of two, but a shorter latency to constriction in the ASD group before this age (de Vries et al. 2021; Dinalankara et al. 2017; Nyström et al. 2015). Kercher et al. (2020) also could not find group differences in latency to constriction. Relative constriction amplitude was found to be larger in the group with increased likelihood of developing ASD, or later in the ASD group by Nyström et al. (2015, 2018). Clearly, with such a range of results in previous studies, it is difficult to interpret our findings in relation to others' and encourage more standardized experimental settings and complementary measures. Furthermore, to investigate the vagal brake theory as referred to in the introduction, the findings need to be correlated to a measure of social communicative functioning.

The group of children with feeding or eating difficulties was very small and was mainly added from an exploratory perspective. To our knowledge, this group has not previously been studied with pupillometry. Against this background, it was no surprise that we could not show any significant differences from the control group, as a lack of power likely influenced our results. During the longitudinal follow-up, some feeding or eating difficulties were also resolved, possibly implying that the concurring altered physiological state also normalized. This should be further investigated in longitudinal studies with larger samples.

Our study has limitations that are important to keep in mind when interpreting our results. First, not all infants participated at all timepoints and at some timepoints, some groups were much smaller than others. Second, the LMM were theory-driven, rather than data-driven, which might have led to other conclusions. The calculated effect sizes (η_p^2) were rather small, which means that not all variance between subjects was explained by a certain factor, even though it differed significantly between groups or timepoints, and that clinical relevance may be modest. Third, pooling of data that was collected with different eye-trackers with different sampling rates at different sites with different environmental luminance, could have introduced extra variability and influenced our results. Especially the latency to constriction parameter could be vulnerable to being measured at different sampling rates. As groups were not evenly distributed over sites, this may have influenced our data. In future research, it is recommended to strive to as minimal differences in equipment and environment as possible. However, due to several steps in our data processing pipeline such as excluding subjects with less than four trials, using averages of the included trials in final analyses, resampling to 300 Hz, using the time to maximum acceleration as a definition of latency to constriction (Bergamin and Kardon 2003; Fish et al. 2021; Nystrom et al. 2015, 2018), we have attempted to minimize potential bias. Data quality as indexed by the proportion of eliminated trials per timepoint and per group, might have influenced our results, since the data quality is clearly poorer at younger age despite our efforts. This result was to be expected, due to the nature of the experiment and the developmental level of infants at 5 months of age (e.g. short attention span, limited motor control, not able to sit independently, …). We need to be cautious when interpreting our results due to this limitation, however we want to encourage to keep including very young infants in future eye-tracking research, despite this limitation, as the developmental trajectories can be very interesting. Group differences in data quality may be related to other factors such as adaptive behavior or developmental level, which should be controlled for in future studies (for example by conducting questionnaires or including observational scores). In summary, group differences that were found might be confounded by data quality and sampling rate, which can best be checked by replicating our research in a single site study and in larger samples.

The pupillary light reflex is an easy-to-measure index of the ANS, which should be further investigated in infants with and without increased likelihood of atypical ANS development, because of the important role of the ANS in regulating the body's physiology when coping with stress. The PLR is a result of signal transmission through several structures and is under the influence of both the PNS and the SNS. When the PLR results differ, we cannot pinpoint anatomically where these differences originate and whether they are an effect of only the PNS or SNS, but we can conclude that the balance within the ANS has shifted. Combining pupillometry with other measures such as heart-rate variability, but also neuroimaging and animal studies, could help us gain further insight into the development of the ANS and the underlying mechanisms and structures that are involved in this process, with the

ultimate goal to facilitate early detection and intervention of atypical ANS development in different groups.

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Author contributions LDV: conceptualization, methodology, data collection, data analysis, writing—original draft, review and editing; SA and TVL: data collection, writing—review and editing; PN: methodology, software, assistance in data analysis, writing—review and editing; LVE: project administration, writing—review and editing; PW and HR and IN: conceptualization, funding acquisition, writing—review and editing; BB: conceptualization, resources, supervision, writing—review and editing; GN and JS: conceptualization, supervision, funding acquisition, writing—review and editing. All authors read and approved the final version of the manuscript.

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Data availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interest.

Ethics approval Approval by the ethical committee at both sites of data collection (Ghent University & KU Leuven) was granted. The research was conducted in accordance with the 1964 Helsinki Declaration.

Informed consent The parents of all participants gave informed consent.

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