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Development of a behavioural test battery for the assessment of pharmacologic and non-pharmacologic environmental factors on the developing brain.

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1 Introduction

1.1 Prematurity and perinatal brain damage

Perinatal brain damage is a leading cause of disability, and even death, in preterm infants. In Germany and Europe, preterm infants comprise 5-11% of all live births (Beck et al., 2010) today. Due to the increasing rates of multiple pregnancies, late motherhood, and infertility treatments, this number is expected to rise in the upcoming years, expanding the surviving population (Keller et al., 2010). In particular, the subpopulation of surviving preterms with very low birth weight (VLBW) ($<$1500 g) increased to approximately 2% of the annual number of births (Martin et al., 2010). This increase poses a serious burden not only to the children and their families but also to health care and, therefore, to society in general. In addition to severe impairments, such as cerebral palsy, it has been shown that several cognitive dysfunctions have a higher incidence in formerly preterm children. Even in children without severe neurodevelopmental impairments, the risk of attention deficit hyperactivity disorder (ADHD) is increased by 2.6–4.0 times in very preterm infants in early childhood. Some of these difficulties persist into adolescence and early adulthood (for review: Saigal and Doyle, 2008). In face of this development, it has become more important to conduct long term studies in clinical and experimental research that investigate the factors causing these sequelae and that scrutinise the safety and consequences of iatrogenic interventions.

1.2 Neurodevelopmental and behavioural changes due to developmental brain injury

Preterm infants are at a higher risk of suffering from neurodevelopmental and behavioural sequelae. With the exception of severe impairments, such as cerebral palsy, it has been shown that several cognitive dysfunctions have a higher incidence in formerly preterm children (for review: Saigal and Doyle, 2008). Several studies reported a higher risk of VLBW infants to suffer from cognitive deficits, leading to academic underachievement, grade failure, and the need for additional remedial assistance during mid-childhood and adolescence (Bhutta et al., 2002, Botting et al., 1998, Breslau et al., 1994, Doyle et al., 2005, Hack et al., 1994, Saigal et al., 2000, 1991, Taylor et al., 2000, Victorian Infant Collaborative Study Group, 1991). Furthermore, the prevalence and severity of these sequelae were found to be especially pronounced in the group of children with the smallest birth weight. Recent reports indicate that these very preterm infants are at a 2.6–4.0 times higher risk of experiencing behavioural problems, such as ADHD (Aylward, 2005, Delobel-Ayoub et al., 2006, Reijneveld et al., 2006), with a special susceptibility to difficulties related to inattention and hyperactivity. In addition, a higher incidence in the experience of emotional troubles at school age has been reported (Anderson et al., 2003, Breslau and Chilcoat, 2000, Sykes et al., 1997), further affecting academic functioning. This pattern is reflected in the observations of most studies (Cooke, 2004, Ericson and Källén, 1998, Hack et al., 2002), indicating slightly lower educational achievements and lower rates of employment and independent living in young adults born VLBW than normal birth weight controls (Cooke, 2004, Ericson and Källén, 1998, Hack et al., 2002).

To date, it is still unclear whether these cognitive deficits alter over time (Aylward, 2005). There have been reports that cognitive disadvantages found in VLBW and extremely low birth weight

(ELBW) infants might persist into late adolescence and early adulthood (Hack et al., 2002, Lefebvre et al., 2005, Saigal et al., 2000). However, a number of environmental factors (e.g., family status, parental socioeconomic status and education, neighbourhood effects, schooling, social and racial backgrounds) also play an important role in cognitive development at this age. Saigal et al. (2006) reported that most of a cohort of advantaged ELBW young adults were able to overcome their difficulties to become functional members of society, despite a high rate of disabilities and educational and behavioural problems encountered in earlier years. This finding strengthens the assumption that social and educational factors play essential roles in the late outcome of cognitive deficits.

1.3 Pathophysiology of perinatal brain injury

The development of brain injury in human newborns is of multi-factorial origin. Several pre- and perinatal factors, such as maternal infection yielding excess inflammation, alterations of cerebral perfusion, arterial stroke, hypoxic-ischaemic injury, growth factor deficiency, oxidative stress, drug exposure, maternal stress, malnutrition, and genetic factors, are likely to play an important role in the aetiology of brain lesions (for review: Ferriero, 2004, Girard et al., 2009). Currently, inflammation and hypoxia-ischaemia are regarded as the two major factors in the aetiology of perinatal brain injury in preterm infants.

Depending on the gestational age, as well as the developmental stage, culprit factors affect grey and white matter structures to varying degrees. A critical feature of neonatal brain damage is that damage occurs at a time when several essential physiological processes occur. In addition to acute damage itself and its accompanied destruction of tissue, mere disturbances in neuronal sprouting, synaptogenesis, selective elimination of neurons, synapse reorganisation, and myelination (see Figure: 1) can also lead to dramatic deterioration of brain function in later life. Magnetic resonance imaging studies revealed that reduced brain volume, reduced cortical folding, delayed maturation, and disturbed myelination are common findings in ex-premature infants, which altogether are associated with an impaired neurological development (Brown et al., 2009).

In addition to these factors, preterms are additionally challenged by their unphysiological postnatal environment, which might interfere with brain development. Higher rates of temperature instability, respiratory distress, apnoea, hypoglycaemia, seizures, jaundice, kernicterus, feeding difficulties, and periventricular leucomalacia (Escobar et al., 2006, Kinney, 2006, Raju, 2006, Wang et al., 2004) in these children somehow reflect their struggle to adapt to their new environment and, of course, increase their need for hospitalisation. This longer hospital stay, however, leads to increases in stressful and even painful procedures, sedation, oxygen supplementation, and additional medication. In recent years, it has become more evident that those so-called environmental factors, such as hyperoxia and drugs, contribute to a further disturbance of brain development (Figure: 2) (Keller and Griesmaier, 2011).

1.4 Propofol in neonatal intensive care

Elective or semi-elective procedures, in addition to other scheduled surgical or investigative procedures requiring sedation, analgesia, or anaesthesia, are commonly performed in neonatal intensive

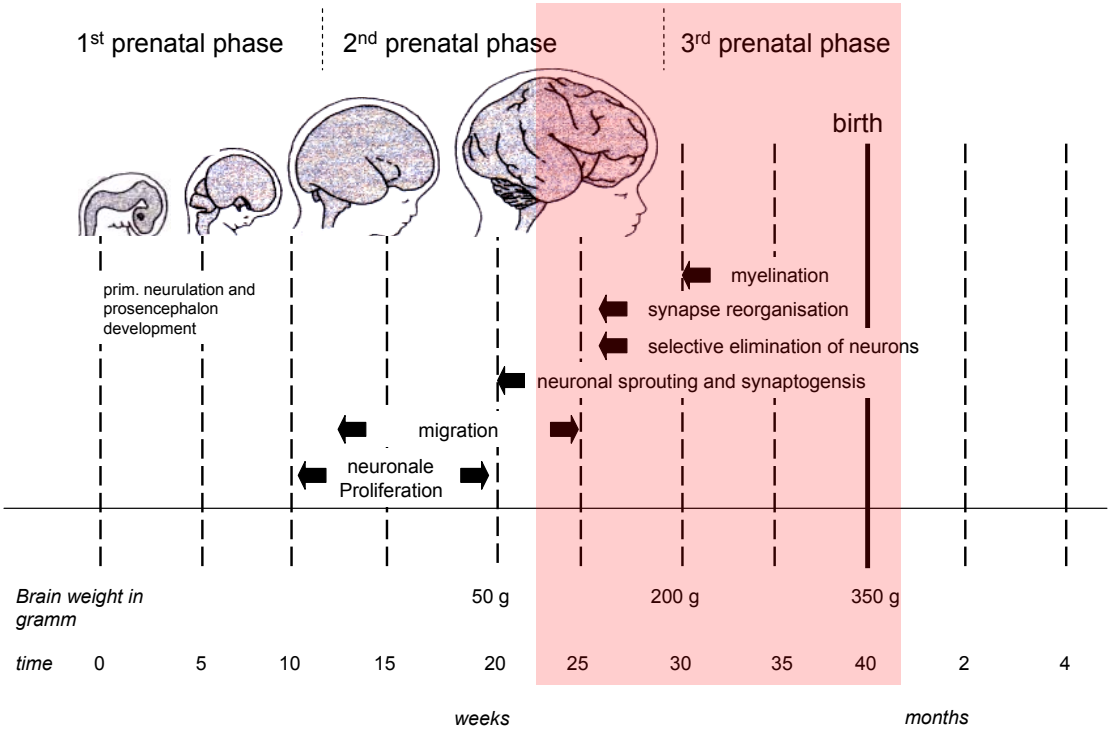

Figure 1: Neonatal brain damage affects essential physiological processes in brain development. Depending on the gestational age, physiological processes such as neuronal sprouting, synaptogenesis, selective elimination of neurons, synapse reorganisation, and myelination are affected to varying degrees, leading to impaired brain function in later life (from: Lagercrantz et al., 2010)

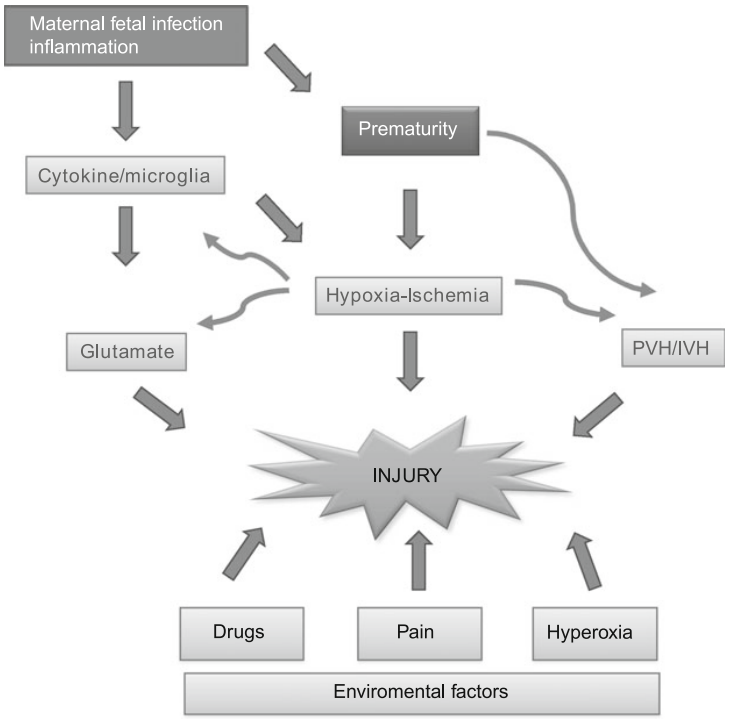

Figure 2: Inflammation, a major cause of preterm birth, triggers microglial cell activation, sensitises to hypoxia-ischaemia and also contributes directly to cell death. Preterm birth, *per se*, is also associated with a greater risk for hypoxia-ischaemia and may result in intraventricular haemorrhage, causing an increase in oxidative stress and excitotoxic cell death by increased levels of glutamate. In addition to these factors, environmental factors such as hyperoxia, drugs, and pain can cause cell death and can induce developmental brain injury (from: Keller and Griesmaier, 2011).

care. These procedures include endotracheal intubation, eye examination for retinopathy of prematurity (ROP) and surgeries such as ligation of patent *ductus arteriosus* or laser therapy for ROP. In current practice, sedative, analgesic and/or anxiolytic agents are administered to reduce pain and stress associated with these procedures and facilitate completion of the procedure in a timely manner (Oei et al., 2002).

Propofol (2,6-diisopropylphenol) is widely used for short-term sedation and anaesthesia in adult and paediatric intensive care units. Introduced as an anaesthetic agent by Kay and Rolly (1977), propofol is a lipophilic, $GABA_A$ mimetic, anaesthetic agent that has a rapid distribution from blood to the central nervous system and adipose tissue and prompt redistribution (Allegaert et al., 2007). Its fast onset of action and quick termination of effects, when discontinued, have attracted wide interest and use.

Similarly to many other sedative, analgesic and/or anxiolytic agents used in neonatal intensive care, propofol is also associated with side effects, including hypoxaemia, bradycardia, hypotension, clonic convulsion (Gelber et al., 1997), and even death in the form of "propofol infusion syndrome" (Bray, 1998).

In 1999, the FDA decreased the approved age for maintenance of anaesthesia with propofol to 2 months, whereas in Germany, the use of 1% propofol is approved for children older than 1 month for induction and maintenance of anaesthesia (Motsch and Roggenbach, 2004). Despite these restrictions, off-label use is common for anaesthesia, even in neonates and preterm infants. This issue has been recently reviewed by Shah and Shah (2011). As a result, no recommendation for the use of propofol in neonates could be given due to the sparse data and the lack of available evidence.

Investigations in animal models indicate that anaesthetics, especially propofol, can induce apoptotic cell death in the brain when administered during synaptogenesis (Bercker et al., 2009, Fredriksson et al., 2007, Pesić et al., 2009, Zacharias et al., 2010).

The mechanisms of the toxic effects on the central nervous system that are induced by anaesthetic agents remain poorly understood. Mitochondrial and death receptor-mediated apoptotic pathways Yon et al. (2005), as well as the brain-derived neurotrophic factor (BDNF)-modulated apoptotic cascade (Lu et al., 2006), have been suggested.

Concerns must be raised that this apoptotic neurodegeneration may lead to behavioural deficits, as it has been already shown for other frequently used substances (e.g., benzodiazepines, barbiturates, volatile anaesthetics). To date, there is no clinical evidence that the use of propofol can cause learning disorders or developmental retardation, although two case reports described neurological sequelae following prolonged propofol infusion (Lanigan et al., 1992, Trotter and Serpell, 1992). Experimental studies have shown that propofol causes apoptosis in the developing brain. The effects on long-term neurodevelopmental outcome, however, remain uncertain.

To the best of our knowledge, only two experimental studies have investigated the long-term cognitive outcome of propofol administration. Using a cumulative dose of 90 mg/kg propofol on six-day-old Wistar rats, Bercker et al. (2009) reported impaired habituation in propofol-treated animals compared to controls at seven weeks of age. Investigating the effects of several subcutaneously administered N-methyl-D-aspartic acid (NMDA) and γ-Aminobutyric acid (GABA) type

A receptor anaesthetic agents, including propofol (10 mg/kg or 60 mg/kg), in ten-day-old NMRI mice, Fredriksson et al. (2007) were not able to detect any effect of propofol administration on behavioural outcome at 55–70 days of age.

1.5 Oxygen in early human development

As the second most abundant element in the Earth's atmosphere, oxygen is critical for aerobic respiration but, due to its high reductive potential, also poses significant dangers to life. Molecular oxygen is reduced to water by the mitochondrial electron transport chain, which thereby enables the conversion of ADP into ATP. This reaction also results in the formation of toxic reactive oxygen species (ROS) that can inflict damage on various biological molecules. In the absence of oxygen, the electron transport chain is inhibited and glucose metabolism is shunted down to the glycolytic pathways, resulting in a depression of cellular metabolism and initiation of apoptotic pathways (Brunelle and Chandel, 2002, Graeber et al., 1996).

The entire process of organogenesis (10–12 weeks of gestation) transpires under hypoxic conditions, without significant maternal blood flow to the foetus. After this point, the placental bed becomes perfused with maternal blood in a pulsatile fashion (Burton et al., 1999, Jaffe et al., 1997). Although foetal haemoglobin has a greater oxygen affinity than maternal haemoglobin, direct measurement of foetal blood oxygen partial pressure (pO_2) indicates a continued hypoxemia throughout the remainder of gestation where foetal arterial and venous pO_2 values rarely exceed ~4 kPa (30 mmHg or ~4% FiO_2) (Emmanouilides et al., 1995), indicating a continuous physiological hypoxia from conception through parturition. After birth, the arterial oxygen tension is increased to 65–80 mmHg (Hoffmann, 2002), even without supplemental oxygen (Castillo et al., 2008). This observation means that preterm infants are exposed to unphysiological hyperoxic conditions.

Although there is important evidence that oxygen therapy in term and preterm neonates should be carefully reconsidered (Higgins et al., 2007, Saugstad, 1988, The BOOST II United Kingdom, Australia, and New Zealand Collaborative Groups, 2013), the optimal oxygen saturation during the first weeks of life is still not defined. A number of studies indicated that a high oxygen saturation ($SaO_2 > 93\%$), or at least higher than 95%, is detrimental. This effect is especially true for ELBW children when compared with a lower saturation (SaO_2 88–93%, or even as low as 85%) (for review: Maltepe and Saugstad, 2009). These studies have shown that a high saturation and fluctuations in SaO_2 lead to significantly more pulmonary problems and an increased rate of severe ROP. Chow et al. (2003) showed that by avoiding both, ROP treatment was almost completely eradicated.

A long-term follow-up study, published by Tin et al. (2001), indicated no detrimental effects of a low saturation regime. In the context of cognitive outcome, one study even demonstrated that maintaining high oxygen saturation targets results in a reduced mental developmental index (Deulofeut et al., 2006).

However, a recent analysis of three international, randomised, controlled trials (The BOOST II United Kingdom, Australia, and New Zealand Collaborative Groups, 2013) revealed that, despite the decreased rate of severe ROP, targeting an oxygen saturation below 90% (SpO_2 85-89%) in

extremely preterm infants was associated with an increased rate of necrotising enterocolitis and death.

Experimental studies on seven-day-old Wistar rats have clearly shown that exposure to 80% oxygen resulted in cell death in the grey matter (GM) and subcortical white matter (WM). This effect could only be observed during this specific state of cortical development, not after exposure at later postnatal ages (Felderhoff-Mueser et al., 2004). Neural cell death was associated with increased cerebral expression of pro-inflammatory cytokines (Felderhoff-Mueser et al., 2005) and a maturation-dependent reduction in myelin basic protein (MBP) (Gerstner et al., 2008). In vitro, 80% oxygen caused caspase-dependent cell death in cultured $O4^{+}O1^{-}$ pre-oligodendrocytes but not in mature $O4^{+}O1^{+}MBP^{+}$ oligodendrocytes (Gerstner et al., 2008).

In a recent extensive investigation, Schmitz et al. (2011) showed that hyperoxia modulates glial interactions involving an alteration in astrocyte glial fibrillary acidic protein (GFAP) and solute carrier family 1, member 3 (SLC1A3) expression and decreased astrocyte mediated glutamate uptake. Furthermore, these changes led to damages in the oligodendroglial lineage and delayed MBP expression. Despite apparent recovery in the glial population and in MBP levels, the disruption in oligodendroglia development and WM maturation, which occur during a critical period of brain development, lead to long-term deficiencies in WM organisation and integrity. These findings indicate that high levels of oxygen cause oligodendroglial and WM damage. However, the overall effect of hyperoxia on the cognitive long-term outcome remains unclear.

1.6 Neurobehavioural sequelae in animal models of perinatal brain injury

One major challenge in neonatal brain research is to evaluate the impact of perinatal brain damage treatment strategies on neurobehavioural outcome.

Experimental studies assessing these long-term cognitive behavioural sequelae have been performed in several animal models of perinatal brain damage, including models of hypoxia-ischaemia (HI) (Fan et al., 2005, Rice et al., 1981), hypoxia (Douglas et al., 2007, Fagel et al., 2006) and excitotoxic brain damage (Marret et al., 1995).

Some studies assessing long-term cognitive effects have been performed using the Rice-Vannucci HI model (Rice et al., 1981). In our own investigation of 120-day-old CD1-mice that were formerly subjected to unilateral common carotid artery ligation or sham operation on P5, we did not determine a particular impairment due to HI (Schlager et al., 2011). Scafidi et al. (2009) suggested that the interpretation of this model might be complicated due to the variability of injury among pups, unilateral ligation and inevitable compensation from the undamaged hemisphere, as well as other unidentified factors. However, using a bilateral common carotid artery occlusion model, Fan et al. (2005) were able to detect a significantly decreased performance in locomotor activity, memory tasks and passive avoidance three weeks after injury. These performances were worsened by increased durations of hypoxia.

Several studies were able to monitor neurodevelopmental sequelae including hyperactivity, increased anxiety (Weiss et al., 2004) and impairment in learning tasks and discrimination (Dell'Anna et al., 1991, Nyakas et al., 1996) in models of chronic perinatal hypoxia. Interestingly, while hyperactivity appears to subside after few weeks, working memory and other impairments seem

to be permanent (Chahboune et al., 2009). Animal models of intermittent hypoxia (P1–P3) showed significant cognitive impairment related to hyperactive behaviour but no attention deficit (Oorschot et al., 2007).

Intracerebral injections of ibotenate to induce excitotoxic brain lesions lead to impairments in odour memory and learning abilities (P6–7) (Bouslama et al., 2006, 2007) and to impairments in memory performance in two- and five-week-old mice (Titomanlio et al., 2010).

Altogether, these models cause disturbances in brain development that mimic the behavioural alterations described in humans to a certain degree; therefore, these models can be used to investigate the effect of interventions on long-term cognitive outcome.

In contrast to these endogenous factors of developmental brain injury, very little is known about the alterations in cognitive performance caused by environmental factors, such as propofol sedation and exposure to unphysiological high levels of oxygen. To supplement our ongoing molecular and histological investigations about the neurodegenerative properties of these environmental factors, we decided to assess the long-term neurobehavioural outcome.

1.6.1 Neurobehavioural tests in rodent animal models

As of this day, a broad range of behavioural tests are available and discussed to evaluate emotional state, general motor and sensory function, as well as cognitive function. The aim of this work was to set up a test battery enabling us to monitor the effects of perinatal interventions on long-term neurodevelopmental sequelae. We have focused on three of these tests to assess general motor function, exploration behaviour, and anxiety, as well as memory formation and conditioning. The choice of these tests was driven by (a) the behavioural changes in formerly preterm children observed in clinical studies, as mentioned above, and (b) the consideration of minimising the spatial requirements of the individual test.

Open field behaviour

Hall (1934, 1936) first connected the behaviour of animals in an open arena to their emotional distress and reactivity. Since then, the approach has evolved and gained huge popularity to become the most widely used test in psychology (Bronikowski et al., 2001). The open field test exploits the inner/intrinsic tendency of rats to explore an unfamiliar environment (Kafkafi et al., 2005). In its most basic set-up, the animals are brought to a plain area and observed over a given period of time (Eilam, 2003). Several parameters quantifying the behaviour of the animal in the arena are extracted and then linked to the status of the animal. Behavioural responses expected from open field test (OF) testing include hyperactivity, exploration behaviour, locomotive activity and anxiety.

Novel object recognition

The novel object recognition test (NOR), first established by Ennaceur and Delacour in 1988 (Bevins and Besheer, 2006, Rutten et al., 2008), is based on the observation of Berlyne (1950) that rats tend to spend more time exploring a novel object than a familiar one.

The NOR is designed as a two-trial, non-spatial, non-aversive memory test, consisting of a "sample" phase and a "choice" phase that are separated by a "test-free" interval, during which the animals are returned to their home cages. During the "sample" phase, two identical objects are presented to the animals in the OF arena. The animals are allowed to explore both objects for ten minutes. In the "choice" phase, one of the objects is replaced by a new one, and the animals are allowed to explore both objects for another five minutes. All objects used are made of biological inert material. The object placement within the open field is illustrated in Figure 3.

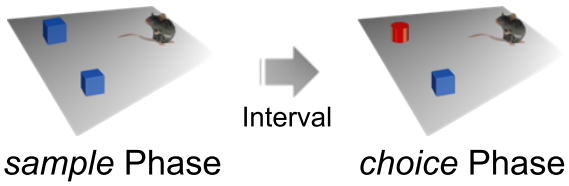

Figure 3: Novel-Object-Recognition Test: spatial arrangement of objects

Modified hole board test

The modified hole board test (mHB), as developed by (Ohl et al., 2001, 1998), is designed as a two-stage test to assess memory-formation and conditioning. It consists of the open field arena described above, containing a board with 10 holes (⌀3 cm; 3 cm deep) that are arranged in two lines. During the first stage, three holes (baited holes) are baited with a small almond piece (0.01 - 0.02 g) placed upon a grid, while the remaining seven cylinders (non-baited holes) contain non-retrievable almond pieces placed beneath the grid. The animal's task is to find the retrievable food rewards without visiting the non-baited holes. This first stage consists of four trials performed on five consecutive days each, with a maximum duration of five minutes or until completion of the task. On the sixth day (second stage), the sequence of baited and non-baited holes is changed compared to the initial stage, where the sequence is kept constant over the four trials. This design enables the performance of spatial tasks and, thus, the investigation of flexible cognitive processes.

2 Study Aim

Preterm infants are at a high risk of suffering from disturbances in brain development with subsequent neurocognitive sequelae. Thus, there is an urgent need to gain insight into the pathophysiology of perinatal brain damage and contributing factors. In addition, there is intensive research on neuroprotective strategies in experimental animal models to alleviate neurocognitive sequelae. Currently, important endpoint parameters consist of histological, molecular biological and biochemical analyses to assess neurodegeneration and neuroprotection. It is, however, of utmost importance to also focus on the functional outcome and, therefore, the long-term behavioural effects. To accomplish this task, this thesis aimed to set up a test battery that covers a broad spectrum of behavioural tests, which did not exist at our institution in this form.

Therefore, the aim of this thesis was to achieve the following:

- set up routines and protocols for the neurobehavioural assessment of long-term cognitive outcomes in rodents by a behavioural test battery.
- implement statistical procedures to analyse the longitudinal data obtained.
- evaluate whether exposure to propofol and hyperoxia during the neonatal period causes long-term deficits.

3 Hypothesis

Environmental factors, such as sedation (propofol) and unphysiological oxygen exposure (hyperoxia), disturb neural development, resulting in an impaired cognitive function later in life that can be detected by common behavioural tests.

4 Materials and Methods

4.1 Animal handling

All animals were housed under standard conditions (water and food *ad libitum*, 12 h day/night cycle, 25°C room temperature (RT), 75% relative air humidity). Procedures and protocols were designed to conform to the European Union Principles of Laboratory Animal Care and were approved under the German Animal Protection Law by both federal and state oversight agencies (Propofol: TSG Nr.: G1113/10; Hyperoxia: TSG Nr.: G1111/10). Animals were originally obtained from Harlan Laboratories, Inc. (Rossdorf, Germany) and internally bred in our Central Animal Laboratory (Zentrales Tierlabor – Universitätsklinikum Essen, Germany). Rats delivered spontaneously after a gestation period of approximately 21 days. Pups were housed with their dams until they reached pubescence and were only being separated for interventions (Section: 4.2.1 and 4.2.2 respectively). At 3 weeks of age (P21), the pups were separated from their mothers and weaned. Male and female animals were separated into group housing cages (3–4 animals per cage) (Eurostandard Type IV, $595 \times 380 \times 200$ mm; floor area 1820 cm^2).

4.2 Animal model

4.2.1 Propofol

To investigate the effects of propofol on long-term neurobehavioural outcome, we followed the protocol described in Zacharias et al. (2010). In short, five-day-old (P5) Wistar rats weighing 10–18.53 g were randomly assigned to one of two groups to be treated with intraperitoneal (i.p.) applications of propofol (n=13; 7 male, 6 female) or sham injections of NaCl 0.9% solution as a control group (n=12; 7 male, 5 female). Altogether, 25 rat pups derived from three litters were investigated, and the number of animals in each experiment varied depending on the size of the individual litter (between 4–11 animals). Propofol doses of 30 mg/kg body weight were given every 90 minutes, up to a cumulative dose of 90 mg/kg. Intraperitoneal injections were performed with a 21-gauge hypodermic needle attached to a 1 ml syringe. After the injection, the needle was left in place for an additional 20 seconds to avoid leakage. Doses of propofol were determined in pilot studies seeking to achieve a depth of anaesthesia with no, or only minor, reaction to a pain stimulus while maintaining sufficient spontaneous breathing and normal skin colour. To prevent hypothermia, animals were placed on a heating device (37°C) during the anaesthetic procedure. All animals were separated from their mothers during the experimental period. After the final injection, the animals were observed for another 90 minutes until they were awake and active and then returned to their mother and housed as described above (Section: 4.1). If bradypnoea occurred, rats received a pain stimulus; if breathing did not restart or resuscitation efforts were necessary, the rats were excluded from the study. In total, 5 animals (4 males, 1 female) had to be excluded from the analysis, of which one died after the first dose of propofol, three died after the second dose (2 males, 1 female), and one died at P8 ($n_{control} = 12$, $n_{propofol} = 8$).

4.2.2 Hyperoxia

The model of preterm exposure to hyperoxic conditions that was used corresponds to one published in Hoehn et al. (2003). Five-day-old Wistar rats, weighing 8–12.5 g, were randomly assigned into two groups and treated as follows: (NO) normoxia (FiO_2 21%) and normal saline (NaCl 0.9%) intra-peritoneal (i.p.) injections (10 µl/g); (HO) hyperoxia (FiO_2 80%) and normal saline i.p. injections[1]. Oxygen exposure was achieved by placing the animals in an oxygen chamber (OxyCycler; Bio-Spherix, New York, New York) with a minimal FiO_2 of 80% for a duration of 24 h (80% O_2, rest nitrogen). Control animals were kept at room air. Following oxygen exposure, the pups were returned to their dams and housed as described above (Section: 4.1). No hyperoxia-associated symptoms were observed in the rat pups, and the mortality rate was 0% in both groups. In total, 12 rat pups from two litters were included in the study ($n_{control} = 6$, $n_{hyperoxia} = 6$).

4.3 Behavioural Tests

Behavioural testing was performed on adolescent animals (P30) to evaluate functional long-term outcome. In the propofol study[2], adult animals (P120) were also included. Activity and anxiety related behaviours were assessed by OF (DeFries et al., 1966, Meer and Raber, 2005) and memory function by NOR (Bevins and Besheer, 2006) (propofol) or by mHB (van der Kooij et al., 2009) (hyperoxia). Our decision to switch to the mHB during the project was dictated by the limitations of NOR in detecting subtle changes in memory and conditioning. Both methods were performed in the OF arena.

One week before the start of the behaviour tests (i.e., P23 or P113), animals were handled every other day during their active phase to become familiarised with the experimenter. Animals from the hyperoxia experiment were given two small almond pieces (0.01-0.02 g) in their home cage to become habituated to the same food reward used in the mHB.

After this week of adaptation, animals were subjected to four days of OF, followed by two days of NOR (propofol) or six days of mHB (hyperoxia). All experiments started one hour after lights-out (18:00 CET; active phase). Between each individual test, the arena was wiped clean with 50% isopropyl alcohol (IPA), and faecal matter was removed. Visits in the mHB were scored by an observer blinded to the treatment groups under red light conditions (λ >600 nm). The inter-trial duration for the mHB for each animal was 60 minutes. Data were collected and processed using a video tracking system (VideoMot2, TSE Systems, Bad Homburg, Germany) and exported for statistical analyses.

4.3.1 Open field behaviour

The animals were placed in the centre of a dimly lit OF apparatus (51×51×40 cm), fabricated from a biologically inert, infrared (IR)-translucent material and placed upon an IR-light-box (TSE

[1] Injections of normal saline were performed to ensure comparability with future studies, possibly investigating the effects on neuroprotective treatments to counteract the effects of hyperoxia.

[2] In the course of the experiment, failures in the automated video tracking system (distortions in the video feed), resulted in the total loss of rearing measurements, and the loss of some trial measurements for individual animals. To a feasible extent, missing information was recovered from the digital versatile disc (DVD) recordings of the original trial.

Systems, Bad Homburg, Germany) that emits IR-light (λ ∼850 nm). The arena was cleaned with 50% IPA after each animal. Movements of the animals were tracked by a video tracking system (VideoMot2, TSE Systems, Bad Homburg, Germany) for five minutes. Activity was assessed by investigating time in motion (locomotion), travelled distance (distance) and the ratio of distance/locomotion (speed). The percentage of time spent in the border area, termed the index of anxiety (AI), reflects anxiety-related behaviour (Braw et al., 2006, Hefner and Holmes, 2007). Behaviour is a constantly changing process (Walsh and Cummins, 1976) rather than a static event. To account for this and the adaptation of the animals to the experiment, OF testing was replicated over four consecutive days.

4.3.2 Novel object recognition

In this study, "test-free" intervals of 6 and 24 h were used. The NOR task was analysed with a video tracking system (VideoMot2, TSE Systems, Bad Homburg, Germany) using "three-point detection", which distinguishes between the head, rear end and "centre of gravity" of the animal, enabling us to detect when the animal is exploring a specific object with its nose. An encounter was defined as a direct interaction with the object. Thus, rearing or sitting on the object was not valued as such. By spending more time with the novel object than the familiar one, the animal demonstrates its ability to remember the familiar object (Ennaceur et al., 2005). The preference for the new object is quantified by the discrimination index (DI) (Sutcliffe et al., 2007), calculated as the ratio of the difference between the time spent with the novel object (t_N) and the time spent with the old object (t_O) to the overall time spent exploring any object:

$$DI = \frac{t_N - t_O}{t_N + t_O} \tag{1}$$

A positive DI value expresses the preference of the animal for the new object and, therefore, its ability to remember the one it has already explored in the previous trial. NOR was performed after OF.

4.3.3 Modified hole board test

Cognitive function in hyperoxia-treated animals was further evaluated using a modified version of the mHB (van der Kooij et al., 2010). Movements of the animals were tracked by a video tracking system (VideoMot2, TSE Systems, Bad Homburg, Germany). The parameters from the mHB test consist of the number of visits to baited and non-baited holes, the number of revisits to baited holes, the time (latency) to complete the trial, and the number of food rewards not retrieved by the animals. Evaluation of the mHB experiment was performed by a trained observer. To attract the animals, all holes were flavoured with vanilla extract dissolved in 40% grain alcohol (V&S Vin & Sprit AB, Stockholm, Sweden).

4.4 Histology

4.4.1 Perfusion

After behavioural testing, all animals were perfused at either P50 (hyperoxia) or P150 (propofol). Animals were euthanised by i.p. injection of 10-20 mg/kgBW phenobarbital (Luminal®, Desitin

Arzneimittel GmbH, Germany). After ceasing of the "toe pinch reflex", each animal was thoracotmised and the heart was dissected. The left ventricle was punctured by insertion of a 19G cannula at the *apex cordis*. To provide sufficient outflow, the right cardiac auricle was cut off, and the descending aorta was clamped right above the diaphragm. The perfusion started with 100 ml 1x phosphate-buffered saline (PBS) (pH 7.4/4°C) at a flow of 1000 ml/h, followed by 300 ml of 4% paraformaldehyde (PFA) in 1x PBS (pH 7.4) at a flow of 1000 ml/h. Afterwards, the animals were decapitated and their brains were extracted. Following perfusion, the brains were immersion-fixed in 20x brain-volume 4% PFA-PBS and stored at 4°C.

4.5 Statistical Analyses

Data analyses and representations were performed within the statistical environment R (R Development Core Team, 2010). Estimation of the treatment and time effects were computed by generalised least squares (GLS) regression (Pinheiro et al., 2009). For assessment of anxiety, all analyses were performed on arcsine transformed data and reported as such without back transformation. Poisson distributed count data from the mHB experiment were modelled using Generalised Estimating Equations (GEE) (Højsgaard et al., 2005). Several specifications of the error term were allowed to include the longitudinal structure (i.e., compound symmetry correlation) and to address potential treatment/time-dependent heteroscedasticity. Model selection followed the protocols published in Zuur et al. (2009). Initial model formulation followed the experimental design strictly, including treatment, trial and day (mHB only) as the main effects and interaction terms reflecting Time $\times$ Treatment-dependent changes. This initial model (i.e., the full model) was further reduced by dropping non-significant interaction terms. Both full and reduced models are given in the supplementary information (Sections: A and B), but only the reduced model was used for the interpretation of the results. Fixed effects under consideration were tested with Wald tests. Time- and treatment-dependent changes are described by the coefficients of the linear model. P-values from post-hoc tests were adjusted according to the Holm-Bonferroni method (Holm, 1979) to account for multiple comparisons (expressed as "q" in the text). Unless stated, all statistics are reported and graphed with $\pm$ standard error (SE).

4.5.1 Linear regression model

In general, a statistical model is the mathematical expression of the relationship between dependent (response) and independent (explanatory) variables. The simplest of these models is the *bivariate* (two variables) linear regression model, which is defined by:

$$Y_i = \alpha + \beta \times X_i + \varepsilon_i \quad \text{where} \quad \varepsilon_i \sim \mathcal{N}(0, \sigma^2) \tag{2}$$

where X_i and Y_i represent the values of the independent variables X and the dependent variable Y taken by the observation i[3]. Equation (2) can be extended to a multiple linear regression for the use of M explanatory variables ,as given by:

[3] To conform to the international standard of statistical notation (ISO 3534-1), we refer to population attributes by capital letters whereas lower-case letters refer to sample attributes. Similarly, Greek letters refer to population attributes and Roman letters refer to their sample counterparts.

$$Y_i = \alpha + \beta_1 \times X_i^{(1)} + \beta_2 \times X_i^{(2)} + \ldots + \beta_M \times X_i^{(M)} + \varepsilon_i \quad \text{where} \quad \varepsilon_i \sim \mathcal{N}(0, \sigma^2) \tag{3}$$

In the scope of our experiments, $X^{(1)}$ is the actual trial id or is coding for the treatment status of the animal: $X_i^{(1)} = 1$ if animal i has received treatment, and $X_i^{(1)} = 0$ if i is allocated to the sham (control) group. The unexplained information is captured by the residuals ε_i, which are assumed to be normally distributed with an expected mean 0 and variance σ^2. The unknown parameters, α and β, are the population intercept and slope. In practice, a taken sample of size T is used to obtain estimators of the model coefficients a and b and their confidence intervals (e.g., by ordinary least square regression, OLS), which are then used to make a statement about the population parameters α and β. To make such a statement, based on a taken sample, we must assume the linearity of the relationship between Y and X and several conditions about the residuals, namely normality, homoscedasticity, and independence (Zuur et al., 2009).

Normality

The assumption of normality requires that Y at each particular value of $X^{(1)}$ is normally distributed. Because b coefficients are found by minimising the residual error, heavy skewness or large deviations in the residual distribution can cause severe bias in the estimation of the βs. Assessing the mere distribution of the raw data Y can be misleading as it contains the effects of the explanatory variables, unless a large number of replicate observations for each X can be provided. Instead we applied a simple linear regression model, and inspected the residuals, as they represent the information that is left over after removing the effects of the explanatory variables rather than explicitly testing for normality. We then confronted the model residuals to the theoretical normal distribution by into the so-called Q-Q plot. The added benefit of this approach, compared to testing for normality, is to spot potential outliers, namely measurements with deviant behaviours from the rest of the population. In our measurements, the assumption of normality could not be made for the AI and the count data obtained from the modified hole board test. In the case of the AI, an arcsine transformation of the dependent variable was performed to approximate normality. The arcsine transformation consists of taking the arcsine (inverse function of sine) of a square root of a number, which must be in the range -1 to 1. The result can therefore range from -$\pi/2$ to $\pi/2$. The distribution of the count data obtained from the modified hole board test necessitated a particular class of regression, implying Poisson distributed residuals.

Homoscedasticity

Heteroscedasticity refers to the fact that the variance (dispersion) of the response Y is not the same at each X value. The main impact of heteroscedasticity, known as violation of homogeneity, is about the estimation of the variance (standard errors) of the estimated coefficients but not the estimated coefficients themselves. Suspect b standard errors can lead to biased hypothesis tests and, consequently, erroneous conclusions. When heteroscedasticity is presumably due to the nature of the measurement itself, a first option is to transform the dependent variable. This is

typical of blood parameters or gene expression data, where higher variances are observed at higher values. Logarithmic and related transformations are commonly applied to alleviate mean–variance dependency at the price of concealing additional biological information. A second approach is to introduce into the regression model a weighting schema based on the suspected sources of heteroscedasticity. In our experiments, systematic variation could be attributed to a particular treatment group or sampling day.

For illustration, let's consider the *bivariate* model 2 where X is a dummy variable coding for two treatment groups ($X = 0$ or $X = 1$) with unequal variances (σ_0^2 and σ_1^2). Stipulating group-dependant heteroscedasticity in the linear model comes down to reformulating the variance structure of random part ε_i of 2 as:

$$Y_i = \alpha + \beta \times X_i + \varepsilon_{ij} \quad \text{where} \quad \varepsilon_{ij} = \mathcal{N}(0, \sigma_j^2) \quad \text{j=0,1} \tag{4}$$

In the OLS framework, this is equivalent to weighting each observation proportionally to the inverse of its variance, $1/\sigma_j^2$. The underlying idea is that observations with small variances provide more reliable estimates of βs than those with larger variances. For every question, such additional variance structure is included in the final/reduced models should the fit be improved in comparison to the model, assuming equal variances within treatment groups. We also provide a plot of the residuals of Y versus the fitted values of Y to identify suspicious variance patterns.

Independence

The most important assumption of the linear regression model is that the individual observations used to estimate the regression coefficients are independent. As multiple measurements are taken on the same animals, the response is expected to be correlated between trials and/or days. As correlation is assumed between residuals at individual observations, the straight application of the standard regression model would lead to incorrect p-values and intervals of confidence (CIs).

In the standard regression 2, the correlation between the residuals from two measurements, i and i', is implicitly given by:

$$cor(\varepsilon_i, \varepsilon_i') = \begin{cases} 1 & \text{if } i = i' \\ 0 & \text{else} \end{cases} \tag{5}$$

or, in matrix notation, to the correlation and covariance matrices:

$$cor(\boldsymbol{\varepsilon}_{i,i'}) = \begin{pmatrix} 1 & 0 & \cdots & 0 \\ 0 & 1 & \cdots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & 0 & \cdots & 1 \end{pmatrix} \quad \Longleftrightarrow \quad cov(\boldsymbol{\varepsilon}_{i,i'}) = \begin{pmatrix} \sigma^2 & 0 & \cdots & 0 \\ 0 & \sigma^2 & \cdots & 0 \\ \vdots & \vdots & \ddots & \vdots \\ 0 & 0 & \cdots & \sigma^2 \end{pmatrix} \tag{6}$$

As for heteroscedasticity, one way to incorporate dependencies between the residuals is by reformulating the error term. From the *bivariate* regression (2), index i is substituted by jk to indicate the k^{th} measurement made from animal j. Equation (2) becomes:

$$Y_{jk} = \alpha + \beta \times X_{jk} + \varepsilon_{jk} \quad \text{where} \quad \varepsilon_{jk} \sim \mathcal{N}(0, \sigma^2) \tag{7}$$

To capture the temporal dependence structure between the observations, off-diagonal elements are substituted by an estimate of the correlation between each k of the same animal j. Its most simple form corresponds to the so-called compound symmetry (or exchangeable) correlation structure. It assumes identical correlation ρ between the residuals regardless of the "temporal distance" between observations. Considering two measurement k and k' made on the same animal j, equation (5) becomes:

$$cor(\varepsilon_{jk}, \varepsilon_{jk'}) = \begin{cases} 1 & \text{if } k = k' \\ \rho & \text{else} \end{cases} \quad \text{where } \rho = \theta/(\theta + \sigma^2) \tag{8}$$

and the equations in (6) become:

$$cor(\boldsymbol{\varepsilon}_{jk,jk'}) = \begin{pmatrix} 1 & \rho & \cdots & \rho \\ \rho & 1 & \cdots & \rho \\ \vdots & \vdots & \ddots & \vdots \\ \rho & \rho & \cdots & 1 \end{pmatrix} \Leftrightarrow cov(\boldsymbol{\varepsilon}_{jk,jk'}) = \begin{pmatrix} \theta + \sigma^2 & \theta & \cdots & \theta \\ \theta & \theta + \sigma^2 & \cdots & \theta \\ \vdots & \vdots & \ddots & \vdots \\ \theta & \theta & \cdots & \theta + \sigma^2 \end{pmatrix} \tag{9}$$

Note that the correlation between two animals is always 0, giving a subject block-like structure to the covariance matrix of 9. This model can be easily extended to more sophisticated correlation structures, where ρ varies with the "temporal distance" between observations. As such, an important class is derived from autoregressive processes (e.g., autoregressive process of order 1, AR1 or autoregressive moving average process, ARMA). On the ground of both model fitting and "manual calculation" of the within-animal correlation, we did not find that the choice of AR1 in lieu of the simpler compound symmetry correlation structure was justified in our data. For consistency, all analyses are therefore performed with the assumption of a common correlation between repeated measures. (this approach is also supported by the common recommendations in longitudinal analysis, where reasonable modelling of the correlation structure is favoured over determination of the best correlation structure (Zuur et al., 2009)).

Akaike Information Criterion

Decisions about the introduction of additional variance structure were made on the basis of the lowest Akaike Information Criterion (AIC) (Akaike, 1974), defined as:

$$AIC = -2 \ln \hat{\theta} + 2k \tag{10}$$

where $\ln \hat{\theta}$ is the maximised log-likelihood estimate for the model parameters, and k is the number of parameters in the model. The minimum AIC estimate is a broadly used, versatile procedure for statistical model identification, as it rewards the goodness of fit while penalising the included number of parameters to discourage over-fitting.

Generalised Estimating Equations (GEE)

GLS is still restricted to situations with normally distributed responses. Therefore, generalised estimating equations (GEE), as part of the larger family of General Linear Models (GLM), were used to handle count responses from the mHB experiments. In short, GLM is a general formulation of the linear regression presented above (e.g., equation 2), where the right-hand side, comprising the independent variables (X), is related to the response variable Y via a so-called link function (η). The correspondence between the formulations for Normal- (case of 2, right) or Poisson- (left) distributed responses Y is given below:

$$\begin{array}{rl} Y_i \sim \mathcal{N}(\mu_i, \sigma^2) & Y_i \sim \mathrm{Pois}(\mu_i) \\ E(Y_i) = \mu_i \text{ and } \mathrm{var}(Y_i) = \sigma^2 & E(Y_i) = \mu_i \text{ and } \mathrm{var}(Y_i) = \mu_i \\ \mu_i = \eta(X_{i1}, \ldots, X_{iq}) & \log(\mu_i) = \eta(X_{i1}, \ldots, X_{iq}) \Leftrightarrow \mu_i = e^{\eta(X_{i1},\ldots,X_{iq})} \end{array} \tag{11}$$

where $E(Y)$ and var(Y) are the expected values and variances of Y and βs of the unknown parameters. η is the identity link (i.e., 1) for normally distributed responses and is the so-called log-link function for Poisson distributed responses. Therefore, one must keep in mind that the coefficient must be exponentiated on basis e to be interpreted in meaningful manner.

Model coefficients table

Time- and treatment-dependent changes are described by the coefficients of the linear regression model, as given in the tables below (1,3, and 6). These tables show the coefficients estimated by GLS or GEE. The first row in each table ("Intercept") gives the estimated mean value for each parameter ± standard error on the first day of observation in the control group. The following rows show how and how much this value is changed by a certain level of an independent variable ± SE. If this change was found to be significant, p-values are indicated at the end of each column as * p<0.05,** p<0.01,*** p=0.001). For example, as given in table (1), we have estimated a mean time in motion of 111 s ±6.69 SE for control animals on P30. This value was reduced on the second day by -12.2 s ±7.04 SE, which was not found to be significant. One day later, the time in motion was decreased by -32.9 s ±6.85 SE, compared to the first day, which was found to be highly significant. In cases where the interaction term was dropped from the final model (e.g., Speed and AI in table 1), the line "propofol" gives the change that has to be added to the intercept if an animal was treated with propofol. Speed on P30 to P34, for example, has been estimated to be increased in each trial by 0.01 s ±0.01 SE in propofol-treated animals. The interaction term included in the model for locomotion and distance on P30–P34 allows for a different change by treatment in each trial. Thus, propofol-treated animals spent 111 s ±6.69 SE + 28.2 s ±10.6 SE in motion during the first trial, whereas they spent 111 s ±6.69 SE +(-12.2 s)±7.04 SE + 28.2 s ±10.6 SE +(-27.5 s)±11.0 SE in motion on the second day.

5 Results

5.1 Propofol

5.1.1 Open field test

To assess the functional outcome of neonatal propofol treatment, we assessed parameters of activity and anxiety-related behaviour in adolescent (P30) and adult (P120) animals by means of an OF test. We report a significant change in the parameters of activity in formerly propofol-treated animals.

Activity is elevated by propofol treatment in adolescent, but not in adult, animals

Analysis of OF activity for propofol-treated animals between P30 and P34 revealed an elevated pattern of general activity compared to the controls (Figure: 4). Propofol-treated animals spent, on average, 12.8 s ($\pm$ 8.24 SE) more time in motion ($F(1,71) = 7.12$, $p = 9.43 \times 10^{-3}$; Figure: 4a), which was also reflected by an increased travel distance (3.87 m $\pm$ 2.08 SE) ($F(1,71) = 7.36$, $p = 8.37 \times 10^{-3}$; Figure: 4b). This increase was most pronounced on the first day of OF, where propofol-treated animals travelled 28.2 sec ($\pm$ 10.6 SE) longer than control animals ($t(71) = 2.67$; $q = 0.038$) and moved an additional distance of 6.67 m ($\pm$ 2.46 m SE) ($t(71) = 2.71$; $q = 0.034$). After the first day, observations in propofol-treated animals dropped to levels comparable to the ones found in control animals. The average velocity of movement (speed) was not significantly different across groups ($F(1,71) = 0.94$, $p = 0.34$; Figure: 4c). Animals of both treatment groups spent most of their time in the border area of the OF, reflected in the index of anxiety (AI). There was no significant difference between propofol-treated and control animals ($F(1,74) = 0.02$, $p = 0.89$; Figure: 4d).

The assessment of OF activity parameters between P120 and P124 (Figure: 5) showed that the alterations estimated in adolescent rats (P30) were not present in adult animals. There were no detectable changes in locomotion ($F(1,74) = 2.49$, $p = 0.12$; Figure: 5a) or travel distance ($F(1,74) = 1.86$, $p = 0.18$; Figure: 5b), as observed in the adolescent animals. The average velocity of movement (speed) was also not significantly altered between groups ($F(1,74) = 0.44$, $p = 0.51$; Figure: 5c) in adult animals (P120). Similar to the estimations in adolescent animals, there were no significant changes in the index of anxiety (AI) in the propofol-treated and control animals (AI_{P120}: $F(1,74) = 1.09$, $p = 0.30$; Figure: 5d).

Propofol-treated and control animals are able to habituate to the testing procedure

As indicated by the model coefficients (Table: 1) and the corresponding Wald-Test statistics (Table: 2), all observed parameters assessed in adolescent rats (P30) changed significantly over repeated trials in a non-linear fashion. Locomotion ($F(3,71) = 13.6$, $p = 4.08 \times 10^{-7}$; Figure: 4a) and distance ($F(3,71) = 5.35$, $p = 2.23 \times 10^{-3}$; Figure: 4b) significantly declined with the number of trials, from P30 to P34. On the other hand, the average velocity of movement ($F(3,74) = 15.7$, $p = 5.53 \times 10^{-8}$; Figure: 4c) and the index of anxiety ($F(3,74) = 7.25$, $p = 2.50 \times 10^{-4}$; Figure: 4d) significantly increased during the same period.

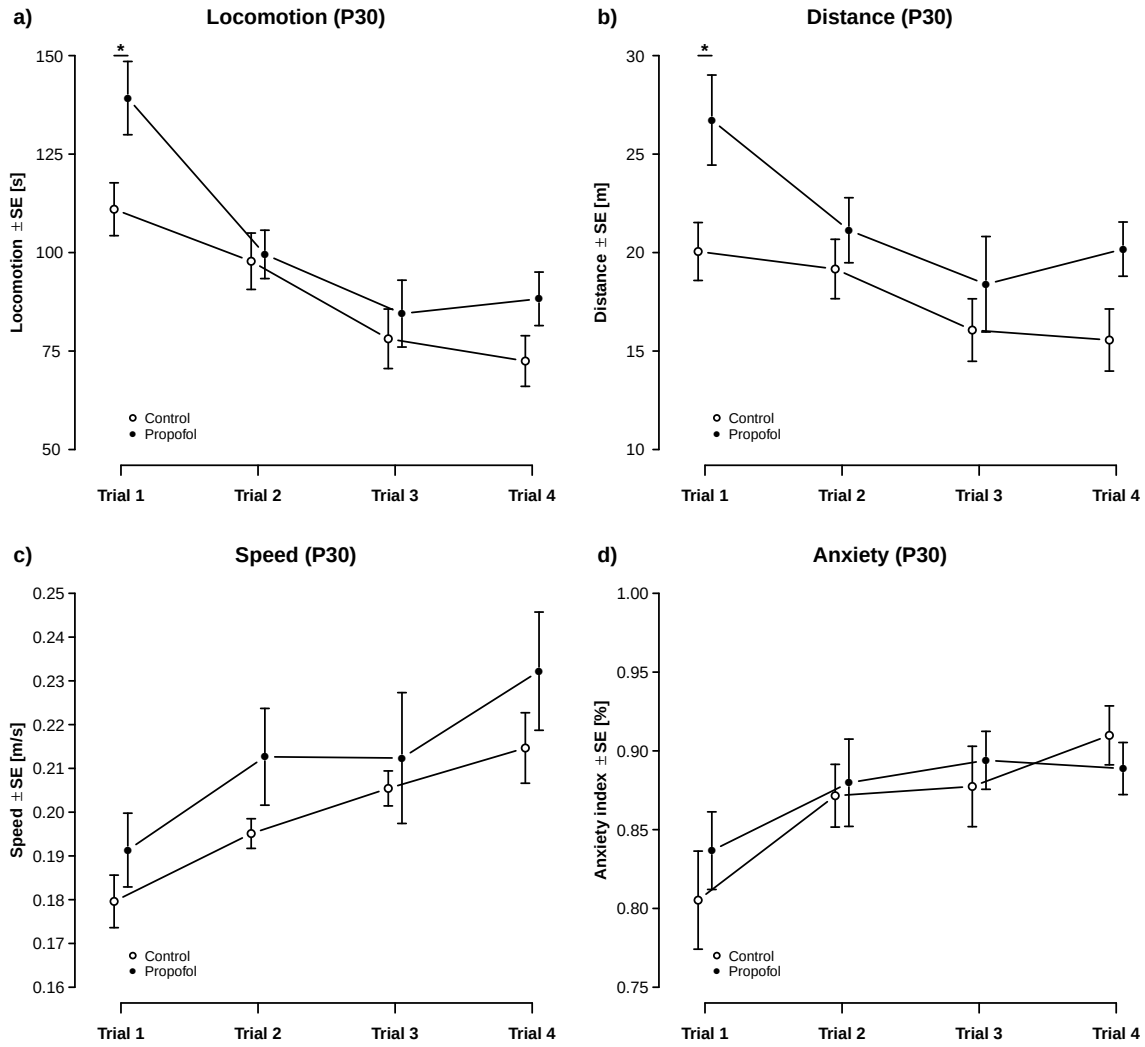

Figure 4: *Propofol - Open field behaviour (P30):* Analysis of activity over 4 repeated measurements points showed overall increases in a) locomotion ($F(1,71) = 7.12$, $p = 9.43 \times 10^{-3}$) and b) distance ($F(1,71) = 7.36$, $p = 8.37 \times 10^{-3}$), but no change in c) speed ($F(1,74) = 1.92$, $p = 1.69$) in propofol-treated animals. An overall change in activity was observed over the individual measurements, resulting in significant decreases in locomotion $F(3,71) = 13.6$, $p = 4.08 \times 10^{-7}$ and distance $F(3,71) = 5.35$, $p = 2.23 \times 10^{-3}$ and a significant increase in speed $F(3,74) = 15.7$, $p = 5.53 \times 10^{-8}$ ($n_{control} = 12$, $n_{propofol} = 8$).

With the exception of a transient increase in locomotion ($F(3,73) = 4.79$, $p = 4.19 \times 10^{-3}$; Figure 5a) on the second and third sampling day, there was no significant trial-dependent alteration in animal behaviour expressed by travel distance ($F(3,74) = 2.56$, $p = 0.06$; Figure: 5b), average velocity of movement ($F(3,74) = 2.25$, $p = 0.09$; Figure: 5c) or the index of anxiety ($F(3,74) = 1.22$, $p = 0.31$; Figure: 5d) in adult animals (P120).

P30–P34

	Locomotion	Distance	Speed	AI
(Intercept)	111.0 ±6.69	20.1 ±1.56	0.18 ±0.01	1.14 ±0.03
Trial 2	-12.2 ±7.04	-0.79 ±1.41	0.02 ±0.01 **	0.08 ±0.03 **
Trial 3	-32.9 ±6.85 ***	-3.99 ±1.37 *	0.02 ±0.01 **	0.10 ±0.03 **
Trial 4	-38.5 ±6.85 ***	-4.50 ±1.37 **	0.04 ±0.01 ***	0.12 ±0.03 ***
propofol	28.2 ±10.6 **	6.67 ±2.46 **	0.01 ±0.01	0.01 ±0.04
Trial 2×propofol	-27.5 ±11.0 *	-4.80 ±2.19 *		
Trial 3×propofol	-21.8 ±10.9 *	-4.35 ±2.16 *		
Trial 4×propofol	-12.4 ±10.8	-2.06 ±2.16		

P120–P124

	Locomotion	Distance	Speed	AI
(Intercept)	32.8 ±4.07	7.24 ±1.21	0.21 ±0.01	1.31 ±0.04
Trial 6	12.0 ±4.17 **	1.52 ±0.67 *	-0.01 ±0.01	-0.04 ±0.03
Trial 7	8.36 ±2.99 **	1.32 ±0.67	-0.01 ±0.01	-0.04 ±0.03
Trial 8	1.71 ±2.95	0.27 ±0.67	0.00 ±0.01	-0.04 ±0.03
propofol	8.59 ±5.44	2.45 ±1.79	0.01 ±0.01	0.05 ±0.05

Table 1: *Propofol - Open field behaviour - GLS Model Coefficients:* All observed parameters showed a significant alteration over repeated measurements. A non-linear decline from P30–P34 was observed for locomotion ($F(3,71) = 13.6$, $p = 4.08 \times 10^{-7}$) and distance ($F(3,71) = 5.35$, $p = 2.23 \times 10^{-3}$). Although there was no overall significant interaction of $trial \times treatment$ in both parameters (Table: 2) the interaction term was kept in the final model to account for the differential behaviour of propofol treated animals on the first day. Both parameters were significantly increased in the frist trial but dropped to levels observed in control animals on the second day of observation. Coefficients are reported ± SE, arcsine transformed values of AI and given without back transformation. ($n_{control} = 12$, $n_{propofol} = 8$) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *(for a general description of the model coefficient table see Section: 4.5.1)*

P30–P34

	Trial	Treatment	Trial×Treatment
Locomotion	F(3,71)=13.6 , p=4.08×10^{-7}	F(1,71)=7.12 , p=9.43×10^{-3}	F(3,71)=2.43 , p=0.0723
Distance	F(3,71)=5.35 , p=2.23×10^{-3}	F(1,71)=7.36 , p=8.37×10^{-3}	F(3,71)=2.09 , p=0.1097
Speed	F(3,74)=15.7 , p=5.53×10^{-8}	F(1,74)=1.92 , p=1.69	
Anxiety	F(3,74)=7.25 , p=2.50×10^{-4}	F(1,74)=0.02 , p=0.89	

P120–P124

	Trial	Treatment
Locomotion	F(3,73)=4.79 , p=4.19×10^{-3}	F(1,74)=2.49 , p=0.12
Distance	F(3,74)=2.56 , p=0.06	F(1,74)=1.86 , p=0.18
Speed	F(3,74)=2.25 , p=0.09	F(1,74)=0.44 , p=0.51
Anxiety	F(3,74)=1.22 , p=0.31	F(1,74)=1.09 , p=0.30

Table 2: *Propofol - Open field behaviour - Wald-Test statistics:* Activity in propofol-treated animals was significantly affected by the treatment with propofol, in terms of increased locomotion and travel distance, in adolescent animals. At 30 days of age, we further determined a significant *trial*-dependent effect in all parameters assessed in adolescent animals (P30–P34). With the exception of a transitory increase in locomotion, we were not able to determine a significant influence of our main effects. Although there was no overall significant interaction of *trial* and *treatment* on locomotion and distance, we kept the interaction term in the final model to account for the differential behaviour of propofol-treated animals on the first day ($n_{control} = 12$, $n_{propofol} = 8$).

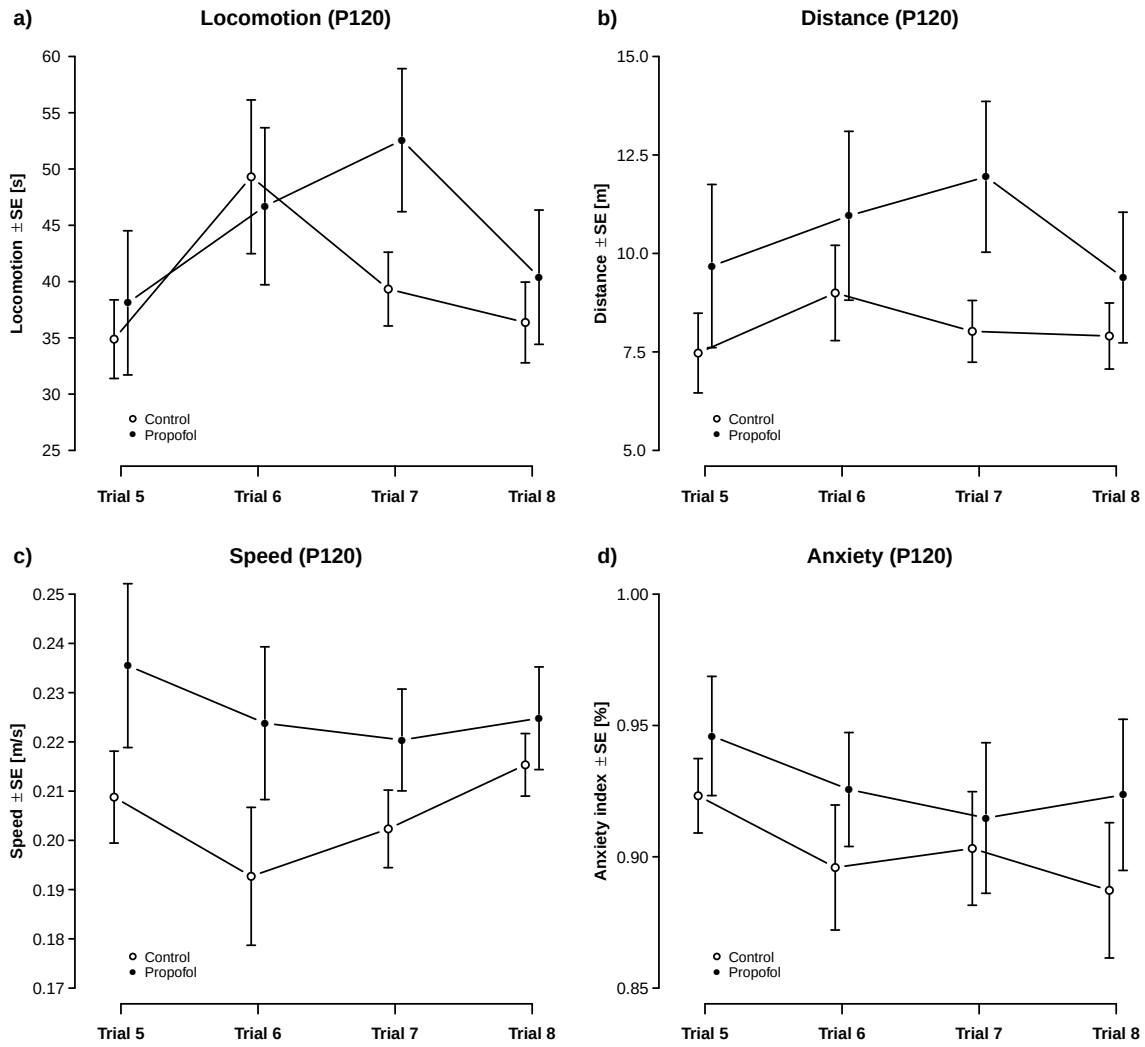

Figure 5: *Propofol - Open field behaviour (P120):* Analysis of activity over 4 repeated measurements points showed no treatment effects on a) locomotion ($F(1,74) = 2.49$, $p = 0.12$) and b) travel distance ($F(1,74) = 1.86$, $p = 0.18$) or c) speed ($F(1,74) = 0.44$, $p = 0.51$) in propofol-treated animals. Apart from a transient effect on locomotion $F(3,74) = 4.79$, $p = 4.19 \times 10^{-3}$, no significant change over repeated measurements was observed in adult animals ($n_{control} = 12$, $n_{propofol} = 8$).

5.1.2 Novel object recognition test

To further assess the effects of neonatal propofol treatment on cognitive performance in terms of object memory, all animals were subjected to NOR following the investigation of activity and anxiety in the OF.

The assessment of memory formation in adolescent animals (P30; Figure: 6a) showed that both propofol-treated animals ($M_{DI} = 0.38,\ SE = 0.12,\ t(7) = 7.45,\ q = 4.3 \times 10^{-4}$) and controls ($M_{DI} = 0.33,\ SE = 0.12,\ t(10) = 6.30,\ q = 3.6 \times 10^{-4}$) spent more time with the new object than with the old object after a 6 h test-free interval. These results indicate that both groups were able to remember the old object over the given time. After increasing the test-free interval to 24 h in the second trial, both the propofol-treated ($M_{DI} = -0.20,\ SE = 0.34,\ t(7) = -1.44,\ q = 0.192$) and the control animals ($M_{DI} = -0.16,\ 0.18,\ t(10) = -1.92,\ q = 0.168$), were no longer able to clearly discriminate between the new and the old object.

In adult animals (P120; Figure: 6b), neither propofol-treated animals ($M_{DI} = -0.10,\ SE = 0.30,\ t(10) = -0.75,\ q = 1.00$) nor controls ($M_{DI} = 0.01,\ SE = 0.28,\ t(7) = 0.10,\ q = 1.00$) spent more time with the new object than with the old object, which again indicates neither group was able to remember the old object. After increasing the test-free interval to 24 h in the second trial, there was also no indication that either propofol-treated ($M_{DI} = -0.09,\ SE = 0.18,\ t(7) = -0.15,\ q = 1.00$) or control animals ($M_{DI} = 0.07,\ SE = 0.11,\ t(10) = -0.18,\ q = 0.789$) were able to remember the old object.

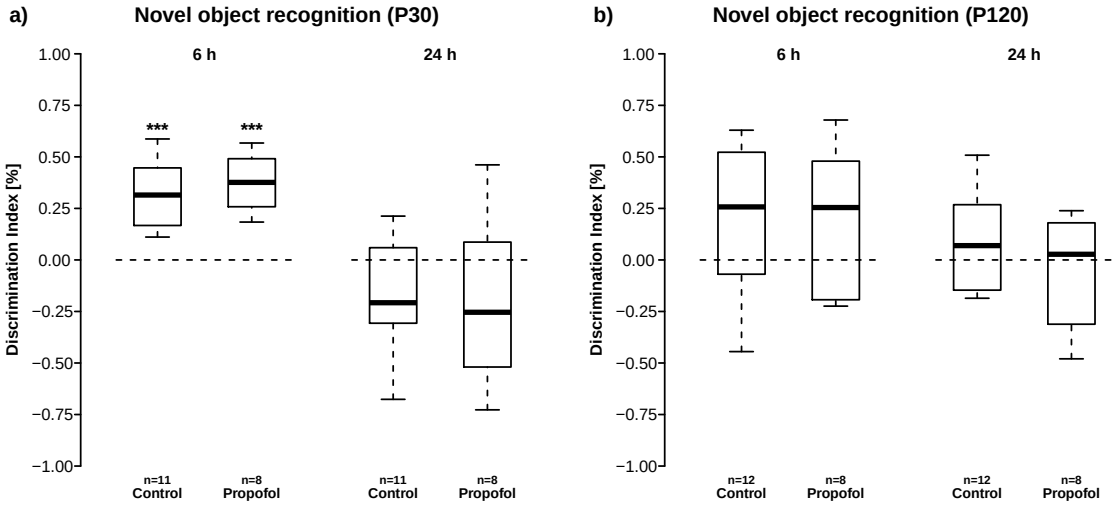

Figure 6: *Propofol - Novel object recognition (P30 & P120):* At the age of 30 days, both propofol-treated animals ($t(7) = 7.45,\ ^{***}q = 4.3{\times}10^{-4}$) and control animals ($t(10) = 6.30,\ ^{***}q = 3.6{\times}10^{-4}$) spent significantly more time with the novel object, indicating their ability to discriminate the novel object from the old object. Propofol ($t(7) = -1.44,\ q = 0.192$) and control animals ($t(10) = -1.92,\ q = 0.168$) failed to do so after a 24 h inter-trial interval. At P120, both groups spent a random amount of time with either of the objects after the 6 h and 24 h intervals, indicating that they were not able to remember the old object.

5.2 Hyperoxia

5.2.1 Open field test

Neither activity nor anxiety are affected by preterm exposure to hyperoxic conditions in adolescent animals

Analysis of OF activity between P30 and P34 in animals that were subjected to 24 h of hyperoxia at P5 revealed no alteration in general activity compared to controls (Figure: 7). Over the observed time, both groups spent similar time in motion ($F(1,43) = 2.86$, $p = 0.098$; Figure: 7a), which, because there was no difference in the average velocity of movements ($F(1,43) = 1.52$, $p = 0.225$; Figure: 7c), corresponded to a similar travelling distance ($F(1,43) = 3.71$, $p = 0.061$; Figure: 7b) in both groups. The estimated number of rearings during the observed time was also not significantly different between hyperoxia-treated and control animals ($F(1,43) = 0.03$, $p = 0.559$; Figure: 7d), indicating that exploration behaviour was not altered by the hyperoxic treatment. Furthermore, there was also no significant difference between the index of anxiety ($F(1,43) = 2.18$, $p = 0.147$; Figure: 7e) in hyperoxia-treated animals compared to controls.

The ability to habituate to the testing procedure is not compromised by preterm exposure to hyperoxic conditions

Similarly to the observations made in the propofol trial (Section 5.1.1), we again estimated a significant change in all assessed parameters over the repeated measurements from P30 to P34. As indicated in tables 3 and 4 and corresponding Figure 7, all parameters changed in a non-linear fashion over the individual trials. We observed a significant decrease in locomotion ($F(3,43) = 10.1$, $p = 3.64 \times 10^{-5}$; Figure: 7a) and distance ($F(3,43) = 3.84$, $p = 0.016$; Figure: 7b), independent of the treatment the animals received. Furthermore, we observed a significant decline in exploration behaviour in both treatment groups, as given by the number of rearings during the measurement ($F(3,43) = 6.39$, $p = 1.12 \times 10^{-3}$; Figure: 7d). The average velocity of movement, in both groups, significantly increased from P30 to P34 in hyperoxia-treated and control animals ($F(3,43) = 16.9$, $p = 2.16 \times 10^{-7}$; Figure: 7c). Overall, the index of anxiety changed marginally over the individual trials ($F(3,43) = 3.04$, $p = 0.039$; Figure: 7e), resulting in a significant increase in the amount of time the animal spent in the border area on the fourth day of observation.

P30

	Locomotion	Distance	Speed	Rearings	Anxiety
(Intercept)	90.2 ±6.53	17.3 ±1.34	0.19 ±0.01	35.1 ±3.11	1.25 ±0.03
Trial 2	-11.2 ±5.45 *	-1.48 ±1.23	0.01 ±0.00	-3.80 ±3.67	-0.03 ±0.03
Trial 3	-17.6 ±5.45 **	-2.13 ±1.23	0.02 ±0.00 ***	-9.20 ±3.67 *	0.00 ±0.03
Trial 4	-29.3 ±5.45 ***	-4.09 ±1.23 **	0.03 ±0.00 ***	-15.1 ±3.67 ***	0.06 ±0.03 *
Hyperoxia	-13.4 ±7.93	-0.01 ±0.00	0.01 ±0.01	-1.80 ±3.04	0.06 ±0.03

Table 3: *Hyperoxia - Open field behaviour - GLS Model Coefficients:* All observed parameters changed significantly over the repeated measurements, especially locomotion, speed, distance, and the number of rearings showed a non-linear decline from P30 to P34, whereas the average velocity of movement and the index of anxiety increased over the same period of time. ($n_{control} = 6$, $n_{hyperoxia} = 6$) $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *(for a general description of the model coefficient table see Section: 4.5.1).*

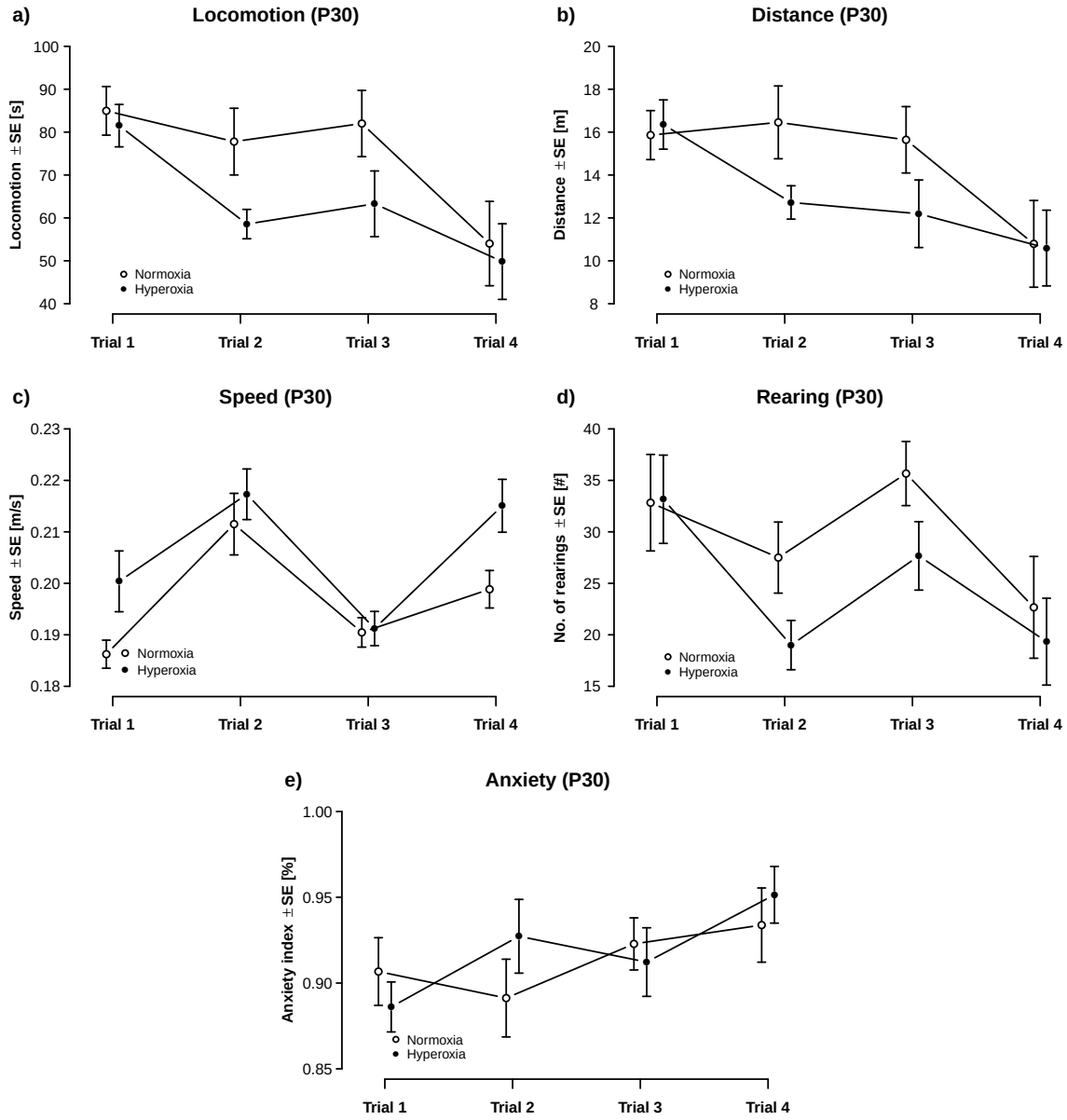

Figure 7: *Hyperoxia - Open field behaviour:* The observation of open field behaviour from P30–P34 revealed that the parameters of locomotion ($F(3,43) = 10.1$, $p = 3.64 \times 10^{-5}$), distance ($F(3,43) = 3.84$, $p = 0.016$), speed ($F(3,43) = 16.9$, $p = 2.16 \times 10^{-7}$), rearing ($F(3,43) = 6.39$, $p = 1.12 \times 10^{-3}$), and anxiety ($F(3,43) = 3.04$, $p = 0.039$) changed significantly over the repeated measurements. We were not able to determine a significant treatment effect in each of the observed parameters ($n_{control} = 6$, $n_{hyperoxia} = 6$).

P30

	Trial	Treatment
Locomotion	F(3,43)=10.1, p=3.64×10^{-05}	F(1,43)=2.86, p=0.098
Distance	F(3,43)=3.84, p=0.016	F(1,43)=3.71, p=0.061
Speed	F(3,43)=16.9, p=2.16×10^{-07}	F(1,43)=1.52, p=0.225
Rearings	F(3,43)=6.39, p=1.12×10^{-03}	F(1,43)=0.03, p=0.559
Anxiety	F(3,43)=3.04, p=0.039	F(1,43)=2.18, p=0.147

Table 4: *Hyperoxia - Open field behaviour - GLS Model Wald-Test statistics:* The observation of open field behaviour revealed a significant change in all observed parameters from P30–P34, which represents the habituation of the animals to the testing procedure. The behaviour of hyperoxia-treated animals did not differ from control animals with respect to our observed parameters of activity, exploration, or anxiety ($n_{control} = 6$, $n_{hyperoxia} = 6$).

5.2.2 Modified hole board test

To investigate cognitive impairment in terms of memory formation in hyperoxia-treated animals, we performed mHB on all individuals following the OF.

Stage 1: Conditioning

In both groups, over all days, the latency to complete the trial was significantly reduced by each trial ($F(1, 232) = 46.1$, $p = 9.20 \times 10^{-11}$). Similarly, the latency was also reduced from day one to day five ($F(4, 232) = 55.6$, $p < 1 \times 10^{-16}$) in the mHB (Figure: 10). In addition, the number of non-baited hole visits also decreased from day 1 to day 5 ($\chi^2(4) = 79.8$, $p = 2.2 \times 10^{-16}$; Figure: 8c). We also estimated a significant change between trials ($\chi^2(1) = 10.4$, $p = 0.0012$) over all days and treatments. However, this change, as indicated by the significant interaction terms, was (i) not the same on each day (Day × Trial: $\chi^2(4) = 16.3$, $p = 0.0026$), and (ii) not the same between treatment groups (Treatment × Trial, $\chi^2(1) = 7.0$, $p = 0.0082$). As illustrated in figure 8c and the model coefficients table (Table: 6), we estimated that there was little to no decline in the overall number of non-baited hole visits between trials, with even a slight increase on the third day of observation, which was reflected in the first interaction term (Day × Trial). In control animals, there was no significant difference in the rate of non-baited hole visits between the first and the last trial on each day. As indicated by the second interaction term (Treatment × Trial), we estimated a steep decline per trial over all days in hyperoxia-treated animals. The rate of non-baited hole visits significantly declined on the first ($\Delta M = -1.01$, $SE = 0.16$, $\chi^2(1) = 37.6$, $q < 1 \times 10^{-16}$), second ($\Delta M = -1.15$, $SE = 0.27$, $\chi^2(1) = 17.4$, $q = 1.18 \times 10^{-4}$) and fourth day ($\Delta M = -0.8668$, $SE = 0.227$, $\chi^2(1) = 14.6$, $q = 4.08 \times 10^{-4}$) of observation. Interestingly, this was not reflected by a significant overall reduction in the number of non-baited hole visits in hyperoxia-treated animals. In contrast to control animals, where no significant difference between the last trial in each day and the first trial on the following day could be estimated, we observed a significant increase in the rate of non-baited hole visits in hyperoxia-treated animals between day one and day two ($\Delta M = 0.54$, $SE = 0.16$, $\chi^2(1) = 11.1$ $q = 3.42 \times 10^{-3}$), as well as between day three and day four ($\Delta M = 0.62$, $SE = 0.21$, $\chi^2(1) = 8.87$, $q = 8.69 \times 10^{-3}$).

In both treatment groups, the number of revisited baited holes also decreased from day 1 to day 5 ($\chi^2(1) = 20.6$, $p = 3.8 \times 10^{-4}$), with no significant reduction between the individual trials over all days ($\chi^2(1) = 1.29$, $p = 0.255$). In the group of hyperoxia-treated animals, the overall number of revisited baited holes decreased by ∼20% ($2^{-0.31 \pm 0.11}$) ($\chi^2(1) = 8.388$, $p = 0.004$; Figure: 9c). Apart from the first trial on the first day, during which three hyperoxia-treated and one control animal did not find the food reward, and the second trial on the first day, during which one hyperoxia-treated animal did not find the food reward, all animals successfully retrieved all of the baits in all of the trials (data not shown).

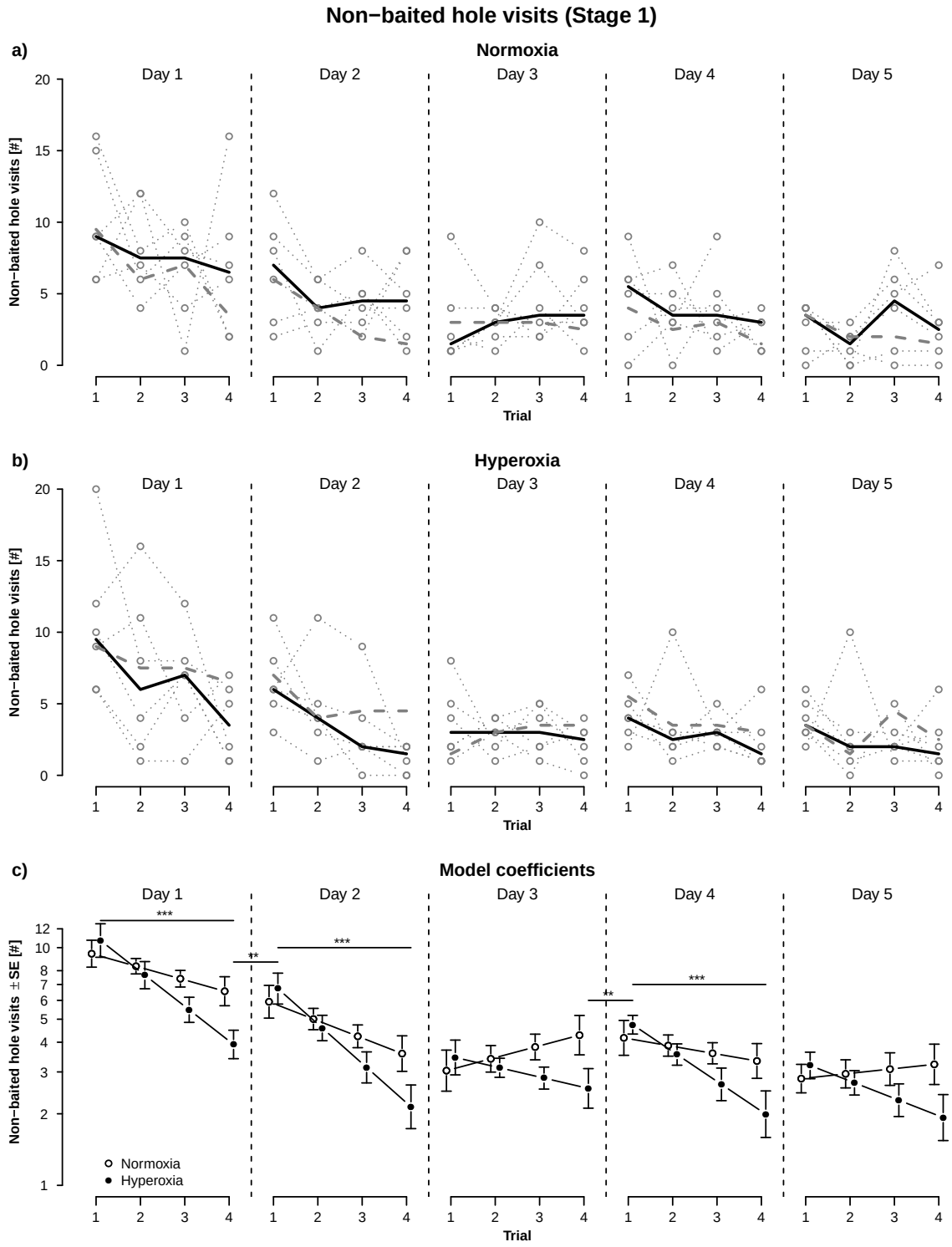

Figure 8: *Hyperoxia - Modified hole board test (Stage 1) - Non-baited hole visits:* In the first stage of mHB, we observed a significant decline in the number of non-baited hole visits ($\chi^2(4) = 79.8$, $p = 2.2 \times 10^{-16}$) from the first to the last day of observation. In hyperoxia-treated animals, we estimated a steeper decline in the number of non-baited hole visits, on each day ($\chi^2(1) = 7.0$, $p = 0.0082$). While there was no significant difference in the rate of non-baited hole visits between the first and the last trial on each day in control animals, the rate of non-baited hole visits in hyperoxia-treated animals significantly declined on the first ($\Delta M = -1.01$, $SE = 0.16$, $\chi^2(1) = 37.6$, $^{***}q < 1 \times 10^{-16}$), second ($\Delta M = -1.15$, $SE = 0.27$, $\chi^2(1) = 17.4$, $^{***}q = 1.18 \times 10^{-4}$) and fourth day ($\Delta M = -0.8668$, $SE = 0.227$, $\chi^2(1) = 14.6$, $^{***}q = 4.08 \times 10^{-4}$) of observation. This was not reflected by a overall reduction in the number of non-baited hole visits, as we observed, in contrast to control animals, a significant increase in the rate of non-baited hole visits in hyperoxia-treated animals between day one and day two ($\Delta M = 0.54$, $SE = 0.16$, $\chi^2(1) = 11.1$ $^{**}q = 3.42 \times 10^{-3}$), as well as between day three and day four ($\Delta M = 0.62$, $SE = 0.21$, $\chi^2(1) = 8.87$, $q =^{**} 8.69 \times 10^{-3}$). Figures a–b) show the observed number of non-baited hole visits for each animal (dotted lines). The median for each group is represented by the full line, whereas the dashed line represents the median of the corresponding group. Figure c) illustrates the model coefficients for non-baited hole visits ($n_{control} = 6$, $n_{hyperoxia} = 6$).

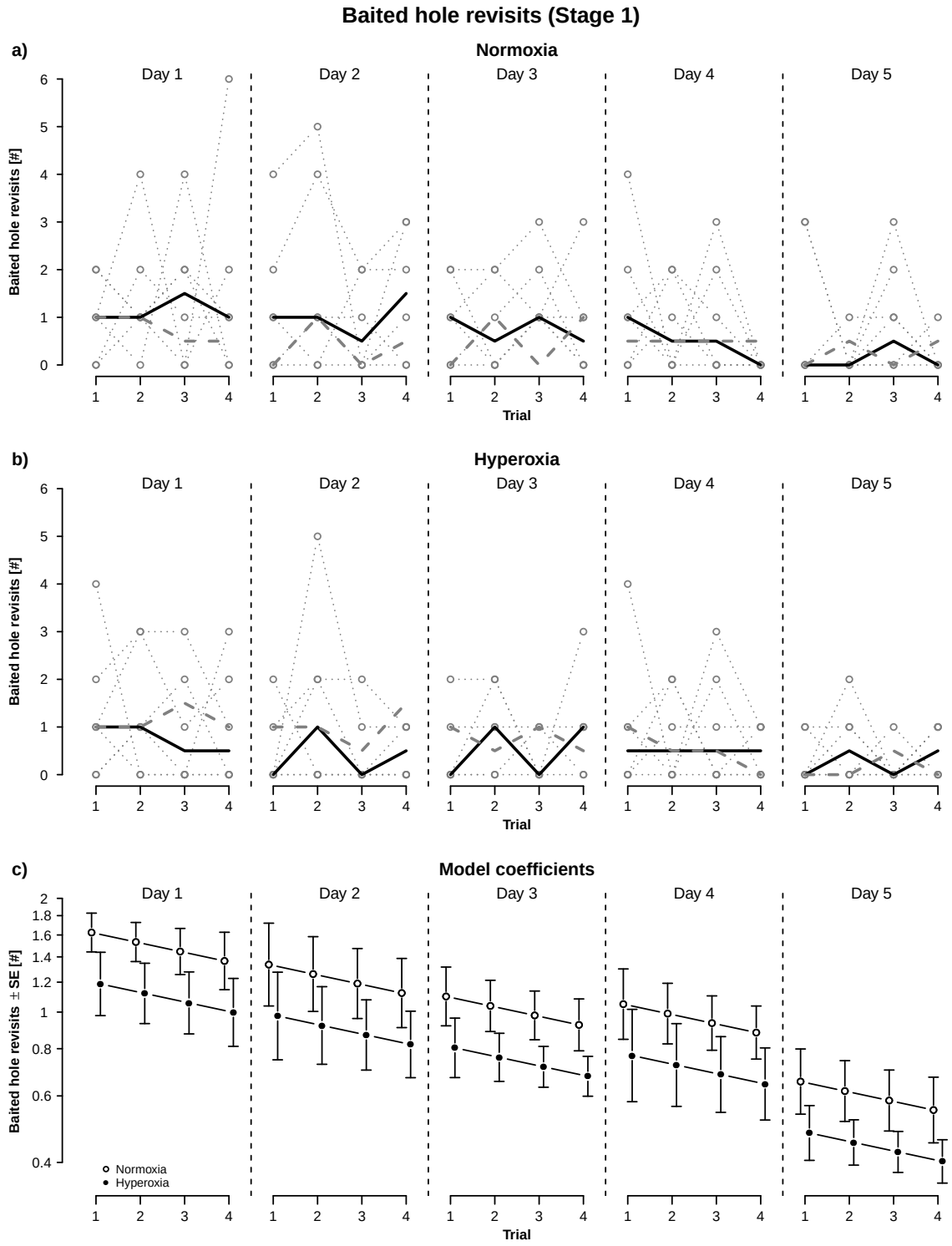

Figure 9: *Hyperoxia - Modified hole board test (Stage 1) - Baited hole revisits:* The number of revisited baited holes, in the conditioning task of the mHB (Stage 1) decreased , in both treatment groups, from day 1 to day 5 ($\chi^2(1) = 20.6$, $p = 3.8 \times 10^{-4}$). In hyperoxia-treated animals, we estimated an overall ∼20% ($2^{-0.31 \pm 0.11}$) reduction in the number of revisited baited holes ($\chi^2(1) = 8.388$, $p = 0.004$). Figures a–b) show the observed number of non-baited hole visits for each animal (dotted lines). The median for each group is represented by the full line, whereas the dashed line represents the median of the corresponding group. Figure c) illustrates the model coefficients for non-baited hole visits ($n_{control} = 6$, $n_{hyperoxia} = 6$).

a) Difference within days

	Control			*Hyperoxia*		
	mean±SE	$\chi^2(1)$	q	mean±SE	$\chi^2(1)$	q
Day 1:	-0.36±0.25	2.07	0.60	-1.01±0.16	37.6	1.00×10^{-4}
Day 2:	-0.50±0.31	2.67	0.51	-1.15±0.27	17.4	1.18×10^{-4}
Day 3:	0.34±0.36	0.92	1.00	-0.30±0.34	0.78	0.38
Day 4:	-0.22±0.32	0.50	1.00	-0.87±0.23	14.6	4.08×10^{-4}
Day 5:	0.14±0.18	0.59	1.00	-0.50±0.27	3.59	0.12

b) Difference between days

	Control			*Hyperoxia*		
	mean±SE	$\chi^2(1)$	q	mean±SE	$\chi^2(1)$	q
Day 1 – Day 2:	-0.10±0.26	0.15	1.00	0.54±0.16	11.1	3.42×10^{-3}
Day 2 – Day 3:	-0.17±0.30	0.30	1.00	0.48±0.32	2.25	0.13
Day 3 – Day 4:	-0.03±0.34	0.01	1.00	0.62±0.21	8.87	8.69×10^{-3}
Day 4 – Day 5:	-0.17±0.24	0.53	1.00	0.47±0.21	4.99	5.08×10^{-2}

Table 5: *Hyperoxia - Modified hole board test - Trial dependent changes in non-baited hole visits:* Assessment of memory function in hyperoxia-treated animals revealed several differences in the rate of non-baited hole visits during stage one of the mHB compared to control animals. On the first, second, and fourth day, the number of non-baited hole visits significantly declined from Trial 1 to Trial 4 in hyperoxia-treated, but not in control, animals (a). However, whereas the rate of non-baited hole visits did not differ between the last trial on each day and the first trial on the following day (b), this rate significantly increased after the first and third day of observation. Mean values and standard errors are given as logarithmic values without back transformation. Corrected p-values are expressed as "q" ($n_{control} = 6$, $n_{hyperoxia} = 6$) .

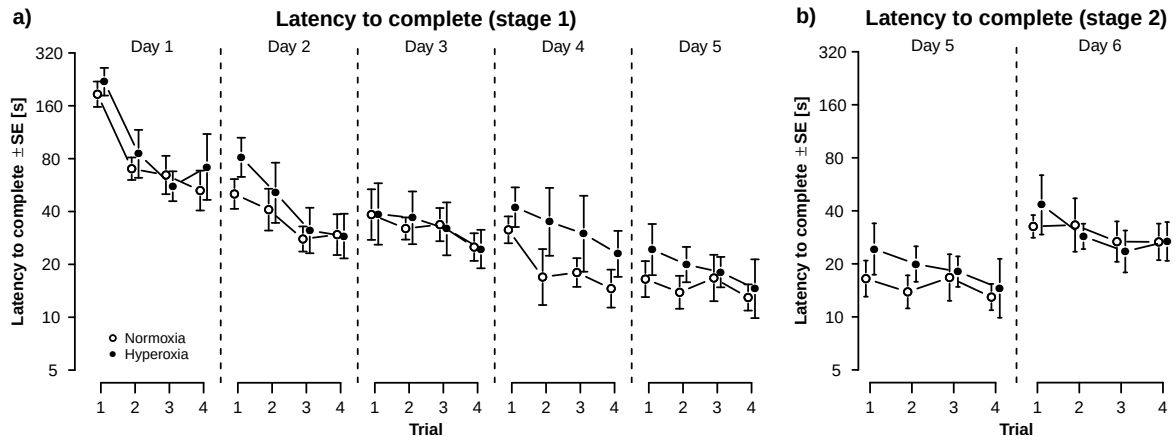

Figure 10: *Hyperoxia - Modified hole board test - Latency to complete:* During stage one of the mHB (a), the latency to complete the trial was significantly reduced by each trial ($F(1, 232) = 46.1$, $p = 9.20 \times 10^{-11}$) and from day 1 to 5 ($F(4, 232) = 55.6$, $p < 1 \times 10^{-16}$), with no significant difference between hyperoxia-treated and control animals ($F(1, 232) = 0.81$, $p = 0.368$). In the second stage, (b) the latency to complete significantly increased from day five to day six $F(1, 92) = 28.8$, $p = 5.89 \times 10^{-7}$, but was reduced by each trial ($F(1, 92) = 5.94$, $p = 0.0167$) before and after the sequence of baited holes was scrambled prior to the first trial on the sixth day of observation ($n_{control} = 6$, $n_{hyperoxia} = 6$).

Stage 2: reconditioning

Further cognitive impairment in hyperoxia-treated animals was investigated by means of a reversal mHB test. During this task, the latency to complete the trial significantly increased in both groups compared to the fifth day of the first stage ($F(1, 92) = 28.8$, $p = 5.89 \times 10^{-07}$) (Figure: 10b). In both groups, the latency to completion was significantly reduced by each trial ($F(1, 92) = 5.94$, $p = 0.0167$). There was no significant difference between hyperoxia-treated and control animals in the latency to complete the task ($F(1, 92) = 0.24$, $p = 0.6243$). Compared to the previous day, we observed a strong increase in the number of non-baited hole visits ($\chi^2(1) = 92.9$, $p < 2 \times 10^{-16}$) in both treatment groups (Figure: 11), with no significant difference between hyperoxia-treated and control animals ($\chi^2(1) = 0.297$, $p = 0.586$). In general, there was no significant change in the number of non-baited hole visits over the individual trials ($\chi^2(1) = 0.302$, $p = 0.583$). However, similar to the observation in stage one, we again detected a steeper decline in the number of non-baited hole visits in the hyperoxia-treated animals in this stage ($\chi^2(1) = 5.38$, $p = 0.02$). The number of baited hole revisits (Figure: 12) also significantly increased during reconditioning, compared to the day before ($\chi^2(1) = 8.47$, $p = 0.0036$). We did not detect a significant change between individual trials ($\chi^2(1) = 0.68$, $p = 0.41$). In contrast to stage one, we did not observe a significant difference between groups ($\chi^2(1) = 0.83$, $p = 0.36$).

stage 1

	Latency	no bait	re. bait
(Intercept)	2.137 ±0.0882	2.3652 ±0.2255	0.5427 ±0.1503
Day 2	-0.339 ±0.0507 ***	-0.4195 ±0.2590	-0.1953 ±0.2818
Day 3	-0.432 ±0.0507 ***	-1.3686 ±0.2922 ***	-0.3895 ±0.1789 *
Day 4	-0.548 ±0.0509 ***	-0.8621 ±0.2275 ***	-0.4363 ±0.2489
Day 5	-0.715 ±0.0507 ***	-1.3775 ±0.2514 ***	-0.9083 ±0.2584 ***
Trial	-0.098 ±0.0144 ***	-0.1212 ±0.0843	-0.0578 ±0.0536
Hyperoxia	0.094 ±0.1046	0.3419 ±0.2344	-0.3134 ±0.1082 **
Day 2×Trial		-0.0461 ±0.1013	
Day 3×Trial		0.2357 ±0.1070 *	
Day 4×Trial		0.0466 ±0.0931	
Day 5×Trial		0.1673 ±0.0959	
Trial×Hyperoxia		-0.2144 ±0.0811 **	

stage 2

	Latency	no bait	re bait
(Intercept)	1.325 ±0.0934 ***	0.7329 ±0.1714 ***	-0.3798 ±0.2896
Day 6	0.248 ±0.0461 ***	0.9759 ±0.1009 ***	0.9400 ±0.3254 **
Trial	-0.050 ±0.0206 **	0.0934 ±0.0851	-0.0765 ±0.0934
Hyperoxia	0.052 ±0.1052	0.7129 ±0.2474 **	-0.1776 ±0.1953
Trial×Hyperoxia		-0.2519 ±0.1086 *	

Table 6: *Hyperoxia mHB: GLS/GEE Model coefficients:* From the first to the fifth day of stage one in the mHB, we observed a significant decline in the latency to complete ($F(4, 232) = 55.6$,$p < 2 \times 10^{-16}$) the number of non-baited hole visits ($\chi^2(4) = 79.8$, $p = 2.2 \times 10^{-16}$) and the rate of revisits to baited holes ($\chi^2(4) = 20.6$, $p = 3.8 \times 10^{-4}$). Hyperoxia treatment did not lead to an overall change in the latency to complete the trial ($F(1, 232) = 0.81$, $p = 0.3680$) or the number of non-baited hole visits ($\chi^2(1) = 1.74$, $p = 0.1874$) but led to a reduction in the number of baited hole revisits ($\chi^2(1) = 8.39$, $p = 3.8 \times 10^{-3}$). We further observed a steeper decline in the rate of non-baited hole visits within each day ($\chi^2(1) = 6.98$, $p = 8.2 \times 10^{-3}$).
During stage two of the experiment, we observed a significant increase in the latency to complete ($F(1, 92) = 28.8$, $p = 5.89 \times 10^{-7}$) and in the rate of visits of non-baited holes ($\chi^2(1) = 92.9$, $p < 2 \times 10^{-16}$) and revisits of baited holes ($\chi^2(1) = 8.47$, $p = 0.0036$) after scrambling the familiar sequence of baited holes. A reduction in the latency to complete was found to be significant within each day ($F(1, 92) = 5.94$, $p = 0.0167$). Similar to the observation during the first stage, we observed a significant reduction in the number of non-baited hole visits by each trial in hyperoxia-treated animals ($\chi^2(1) = 5.38$, $p = 0.0204$). ($n_{control} = 6$, $n_{hyperoxia} = 6$) $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ *(for a general description of the model coefficient table see Section: 4.5.1).*

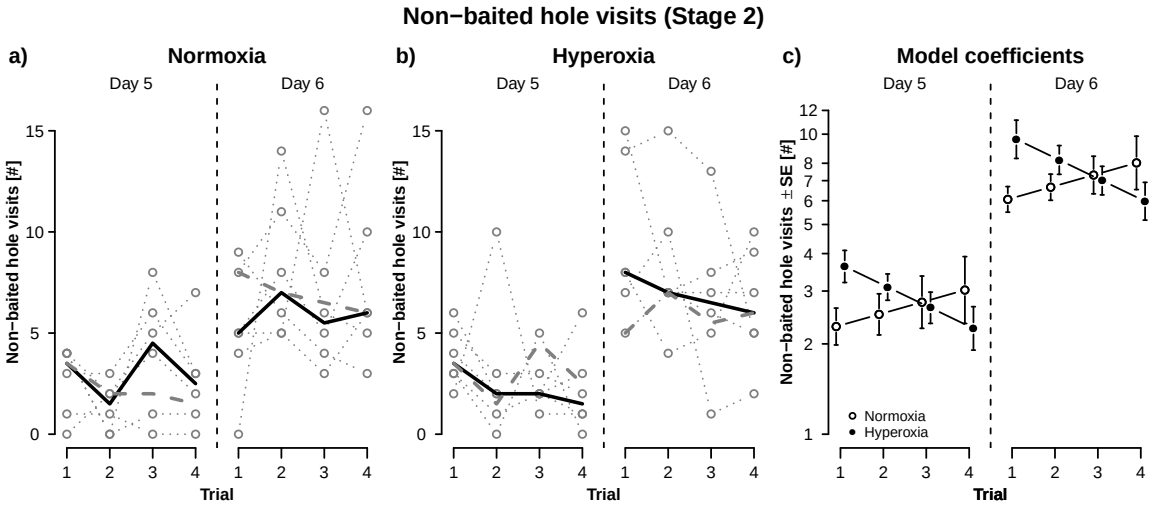

Figure 11: *Hyperoxia - Modified hole board test (Stage 2) - Non-baited hole visits:* In the second stage of mHB, we observed a significant increase in the number of non-baited hole visits after the sequence of baited holes was scrambled ($\chi^2(1) = 92.9$, $p < 2 \times 10^{-16}$), with no significant difference between hyperoxia-treated and control animals ($\chi^2(1) = 0.297$, $p = 0.586$). Although we did not observe a significant decline over the individual trials ($\chi^2(1) = 0.302$, $p = 0.583$), we detected a steeper decline in the number of non-baited hole visits in hyperoxia-treated animals ($\chi^2(1) = 5.38$, $p = 0.02$). Figures a–b) show the observed number, and Figure c) shows an illustration of the estimated model coefficients of non-baited hole visits before (Day 5) and after (Day 6) the sequence of baited holes was scrambled. In figures a–b), the median number of visits for each group is represented by the full line, whereas the dashed line represents the median of the corresponding group ($n_{control} = 6$, $n_{hyperoxia} = 6$).

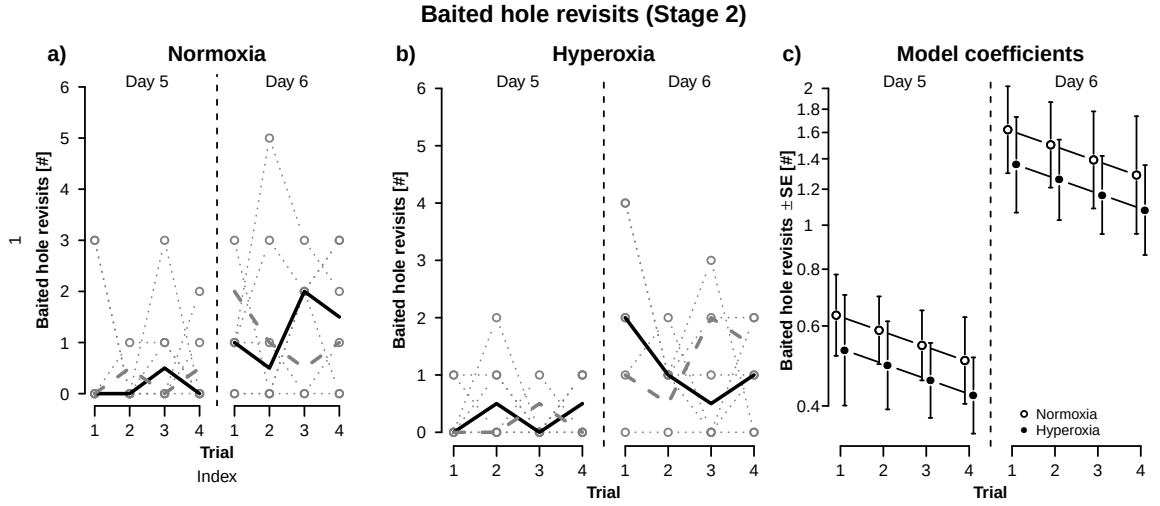

Figure 12: *Hyperoxia - Modified hole board test (Stage 2) - Baited hole revisits:* In the reconditioning task of the mHB, we observed a significant increase in the rate of revisits to baited holes ($\chi^2(1) = 8.47$, $p = 0.0036$) after the bait was retrieved by the animal, with no significant difference between hyperoxia-treated and control animals ($\chi^2(1) = 0.83$, $p = 0.36$). Figures a–b) show the observed number, and figure c) shows an illustration of the estimated model coefficients of baited hole revisits, before (Day 5) and after (Day 6) the sequence of baited holes was scrambled. In figures a–b), the median number of visits for each group is represented by the full line, whereas the dashed line represents the median of the corresponding group ($n_{control} = 6$, $n_{hyperoxia} = 6$).

stage 1

	Latency	no bait	re. bait
Day	F(4,232)=55.6, p<2×10^{-16}	χ^2(4)=79.8, p=2.2×10^{-16}	χ^2(4)=20.6, p=0.00038
Trial	F(1,232)=46.1, p=9.20×10^{-11}	χ^2(1)=10.4, p=0.0012	χ^2(1)=1.29, p=0.25535
Treatment	F(1,232)=0.81, p=0.3680	χ^2(1)=1.74, p=0.1874	χ^2(1)=8.39, p=0.00378
Day×Trial		χ^2(4)=16.3, p=0.0026	
Trial×Hyperoxia		χ^2(1)=6.98, p=0.0082	

stage 2

	Latency	no bait	re bait
Day 6	F(1,92)=28.8, p=5.89×10^{-7}	χ^2(1)=92.9, p<2×10^{-16}	χ^2(1)=8.47, p=0.0036
Trial	F(1,92)=5.94, p=0.0167	χ^2(1)=0.30, p=0.5827	χ^2(1)=0.68, p=0.4088
Hyperoxia	F(1,92)=0.24, p=0.6243	χ^2(1)=0.30, p=0.5860	χ^2(1)=0.83, p=0.3631
Trial×Hyperoxia		χ^2(1)=5.38, p=0.0204	

Table 7: *Hyperoxia: GLS/GEE Model Wald test statistics:* During the first stage of mHB, we observed a significant reduction in the latency to complete the task, a reduction in the rate of non-baited hole visits and in the rate of baited hole revisits over the repeated measurements. In the rate of non-baited hole visits, we further estimated a significant interaction of (i) Day × Trial and (ii) Trial × Hyperoxia, indicating (1) a different change over repeated trials on each day, and (2) a steeper decline per trial in the number of non-baited hole visits in hyperoxia-treated animals over all days. In the group of hyperoxia-treated animals all observed parameters increased significantly after the sequence of baited holes was scrambled during stage 2, with no signifficant difference between treatment groups. The decrease in the rate of non-baited hole visits by trial was significantly higher than in control animals ($n_{control} = 6$, $n_{hyperoxia} = 6$).

6 Discussion

In the present thesis, we aimed to set up a platform for testing the neurobehavioural phenotype of rodent animal models of preterm brain injury. We have successfully set up several routines and procedures, including testing routines, as well as routines for data processing and statistical analyses. To test this platform, we analysed whether the environmental factors of sedation (propofol) and unphysiological oxygen exposure (hyperoxia) disturb neural development in a way that results in an impaired cognitive function in later life that can be detected by common behavioural tests. The neurobehavioural assessments in both models were conducted in addition to current *in vivo* and *in vitro* studies conducted in our laboratory.

6.1 Behavioural testing

By setting up a basic platform for behavioural testing in our research group, we are now able to extend our research beyond important endpoint parameters, such as histological, molecular biological and biochemical analyses to assess neurodegeneration and neuroprotection, by assessing the functional outcome and, therefore, long-term behavioural effects. However, several additions to these methods might become necessary to assess a comprehensive picture of the neurobehavioural phenotype of our used animal models. As indicated above, our choice of test paradigms was also influenced by the spatial requirements of these tests. Common neurobehavioural tests such as the Elevated Plus Maze, the Zero Maze to assess anxiety, and especially the Morris Water Maze and the Barnes Maze, which are used for assessment of memory function, require considerably more room than the average Open Field arena, not only during testing but also for storage of the apparatus. Fortunately, it was possible to start up an interdisciplinary cooperation under the supervision of the head of the Central Animal Laboratory, Prof. Dr. rer. nat. Gero Hilken, which will enable us to extend our current spectrum of behavioural tests and provide the required infrastructure for these experiments.

6.2 Statistical analyses

The choice of the modelling approaches was driven by several factors that include constraints imposed by the design structure, the repeated nature of the experiments, the dearth of a priori knowledge about the model shape, and the diversity in the measurement types. Repeated measures ANOVA and its non-parametric pendant (Friedmann's test) are the methods of choice for comparing group means/medians across repeated measurements. However, these approaches suffer from severe limitations that are not just restricted to their strong assumptions about the data that are unlikely to stand in our context. The respective methodologies rely on some form of "average" view of the data and, therefore, would fail to identify important spatiotemporal relationships with the behavioural response (Krueger and Tian, 2004). In comparison, performing the data analysis in the more general framework of linear modelling not only alleviates some of the restrictions of ANOVA but also it allows a more flexible formulation of the problem by providing answers to more pertinent questions.

6.2.1 Generalised Least Squares (GLS) and Generalised Estimating Equations (GEE)

To include the extensions mentioned above into the linear regression model, we estimated the environment effects on animal behaviour by generalised least square regression (GLS). GLS is adapted to a broader range of situations, as it allows a more general specification, describing heteroscedasticity and correlation between residuals. GLS can estimate the model coefficients, a and b, together with the weights of the variance per stratum (Section: 4.5.1) and within-subject correlation coefficient, ρ. Yet GLS is still restricted to situations with normally distributed responses. This was the case for all measurements (AI being appropriately transformed), with the exception of the count data from the mHB experiments. For the latter case, we preferred generalised estimating equations (GEE), as it best handles count response with repeated measurements, given the restricted sample size of our data-sets (Højsgaard et al., 2005). Whereas the objectives and the interpretation of GEE models are conceptually similar to those of OLS/GLS, the approach to derive a and b follows a different strategy. In the scope of this thesis, the incentive to use GEE in lieu of GLS resides in the direct connection of GEE to the larger family of General Linear Models (GLM), which has the ability to cope with Poisson response.

6.3 Propofol

Reports about neurological sequelae following propofol infusion (Lanigan et al., 1992, Trotter and Serpell, 1992), the lack of clinical evidence (Shah and Shah, 2011), and the neurodegenerative properties of propofol in animal experiments (Bercker et al., 2009, Fredriksson et al., 2007, Karen et al., 2013, Pesić et al., 2009, Zacharias et al., 2010) have raised considerable doubt about the safety of this drug.

In combination with a study, assessing possible mechanisms of propofol-induced neurotoxicity (Karen et al., 2013), we investigated the long-term consequences of these changes in adolescent (P30) and adult animals (P120). A cumulative dose of 90 mg/kg propofol increased locomotive activity in 30-day-old adolescent animals on the first day of observation. This effect was expressed by an increase in the time the animals spent in motion, which led to an increased travel distance. The average velocity of movement was not significantly altered between treated and control animals, indicating that the observed increase in travel distance on P30 was mainly influenced by an increased time in motion, rather than an increase in travel speed in propofol-treated animals. We suggest that this heightened activity was triggered by the novel environment, as parameters dropped to levels found in control animals after this day and were not significantly different on the following days. The idea of an isolated incident on the first day of testing is further supported by our observation of open field behaviour from P120 to P124, which also revealed no alteration in these parameters. As both treatment groups spent most of their time in the border area of the OF, we conclude that propofol treatment did not lead to a disturbance in anxiety-related behaviour at P30 or on P120.

Our observed animals had no difficulty habituating to the testing procedure. At 30 days of age, both groups showed significant declines in locomotion and travel distance from the first to the last day of observation. Over the same time period, both groups showed significant increases in their average velocity of movement and index of anxiety. These changes reflect the initial

exploration of the unfamiliar environment, which is gradually reduced by repeating the testing procedure. With the exception of a transient increase in locomotion on the second and third sampling day, we observed no trial-dependent change in our observed parameters in adult animals at P120. As both treatment groups at P30 and P120 showed similar changes in parameters observed over repeated measurements, we cannot conclude that either group had a significant inability to habituate to the testing procedure.

This finding stands in contrast to the work from Bercker et al. (2009), who showed a disturbance in the animal's ability to habituate to the test procedure from the first to the second day of the hole-board test when treated with propofol. However, it has to be taken into consideration that Bercker et al. (2009) not only used a different test for habituation, but also used animals at seven weeks of age (P49). It is well known that results obtained from behavioural testing conducted in different laboratories can be substantially different, even if the same tests and standardised protocols are enforced (Wahlsten et al., 2003). Fredriksson et al. (2007) showed in a murine model (ten-day-old NMRI mice) that the administration of neither 10 mg/kg propofol nor 60 mg/kg propofol on the tenth day after birth resulted in a significant alteration of animal behaviour 55 days after birth.

In our study, anaesthesia with propofol on P6 did not result in memory deficits on P30 or on P120. At the age of 30 days, both groups spent significantly more time exploring the novel object than the familiar one presented to them 6 h before. This observation shows that neither group had any problem remembering the old object. Following an increased interval of 24 h, neither group showed this ability; whereas some animals still showed a slight preference for the novel object, others favoured the familiar one. This result indicates that the animals explored both objects in a random manner and thus were not able to discriminate between the objects. This finding stands in accordance with previous results obtained by Bercker et al. (2009) and Fredriksson et al. (2007). Even by using different tests, species and time points, none of the results indicate an impairment in memory functioning in propofol-treated animals, which stands in clear contrast to treatment with other clinically relevant anaesthetic drugs. A cocktail of midazolam, nitrous oxide, and isoflurane administered to maintain anaesthesia for 6 hours caused widespread neurodegeneration in the developing brain and neurocognitive deficits that persisted throughout adolescence into adulthood (Jevtovic-Todorovic et al., 2003). The authors proposed that the anaesthesia-exposed rats had lasting deficits in hippocampal synaptic function and, furthermore, that memory functions are mediated by a distributed network that includes the hippocampus, anterior thalamic nuclei, mammillary bodies, and retrosplenial cortex. Each of the latter three structures was damaged more severely than the hippocampus in the anaesthesia-exposed brains (Jevtovic-Todorovic et al., 2003). Similar findings have been described after neonatal exposure to isoflurane alone (Stratmann et al., 2009) and propofol when the Morris Water Maze Test was applied (Bercker et al., 2009). Fredriksson et al. (2004, 2007) exposed infant mice to the NMDA antagonist ketamine or to GABA agonists (diazepam, thiopental and propofol) and demonstrated that these drugs, especially if used in combination, can cause long-term locomotor and cognitive deficits. Interestingly, however, no significant effects on spontaneous behaviour or habituation were observed when these mice were exposed to propofol or thiopental alone (Fredriksson et al., 2007).

This work suggests that the observed acute propofol-induced neurodegeneration, combined with a transient disturbance in neurotrophin availability in the thalamus and cortex (Karen et al., 2013), has no long-term effects on cognitive performance in this model.

6.4 Hyperoxia

In addition to iatrogenic interventions, preterms are also challenged by their environment. It has been shown that preterm exposure to atmospheric, or even higher, levels of oxygen affects early postnatal brain development (Castillo et al., 2008, Hoffmann, 2002). To date, the overall effects of hyperoxia on the cognitive long-term outcome remain unclear. In this study, we investigate the long-term consequences of 24 h hyperoxia from P5 to P6 on 30-day-old Wistar rats.

The assessment of OF behaviour revealed no significant effect of hyperoxic environmental conditions in terms of activity, exploration, and anxiety-related behaviour. General activity and exploration behaviour, as reflected by the time in motion, the average velocity of movement, the travelling distance, and the number of rearings, was not altered by the hyperoxic treatment. There was also no disturbance in anxiety-related behaviour. Additionally, we observed that both groups were able to habituate to the testing procedure, as expressed by declines in locomotion, travel distance, and rearing and increases in the average velocity of movement and anxiety-related behaviour from P30–P34.

In our investigation of memory function, we report a significant difference in the behaviour of our observed animals. In both groups, over all days, the latency to complete the trial and the number of non-baited hole visits decreased from day 1 to day 5, which indicates that all animals were able to find the food reward well. In hyperoxia-treated animals, we observed a significant reduction in the overall rate of baited hole revisits, which indicates an increased ability to remember these holes during a single trial. We further detected a steeper decline in the rate of non-baited hole visits in hyperoxia-treated animals on a trial-to-trial basis within each day, which suggests that these animals became more efficient at finding the baited holes. However, whereas control animals showed a rather steady decline in their rate of non-baited hole visits, with no significant difference between the last observation on each day and the first observation on the following day, we observed a *"sawtooth"* like pattern in hyperoxia-treated animals, with a significant increase in non-baited hole visits. Therefore, we hypothesise that there is an impairment in intermediate to long-term memory in hyperoxia-treated animals, as they were not able to remember the non-baited holes from one day to another. In general, both treatment groups improved in their efficiency to retrieve the baits. However, the method by which this improved efficiency was achieved differed between hyperoxia-treated animals and controls.

Unfortunately, there are currently few to no data available on molecular or histological changes in brain areas involved in the neuroanatomy of memory. Although it has been shown that hyperoxia triggers apoptotic neurodegeneration in the developing rodent brain, which is associated with increases in caspase-1 and the proinflammatory interleukins IL-1β and IL-18 (Felderhoff-Mueser et al., 2005), there are far less data on other regions of the brain such as the hippocampus, the amygdala, or the mammillary bodies of animals exposed to hyperoxia.

Zusammenfassung

Hintergrund: Perinatale Hirnschäden sind einer der führenden Faktoren neonataler Morbidität und Mortalität. Frühgeborene haben ein erhöhtes Risiko, durch eine Störung ihrer Hirnentwicklung, neurologische Spätfolgen davonzutragen. Abhängig vom Ausmaß dieser Störung, reicht das Spektrum dieser Spätfolgen von der Entwicklung einer spastischen Cerebralparese bis zu neurologischen Defiziten wie dem Aufmerksamkeitsdefizit-/Hyperaktivitätssyndrom. Die Pathophysiologie des neonatalen Hirnschadens ist aktuell Gegenstand wissenschaftlicher Untersuchungen. Neben histologischen, molekularbiologischen und biochemischen Analysen ist die Untersuchung des funktionellen Outcomes und somit des Verhaltensphänotyps von entscheidender Bedeutung.

Ziele: Ziel dieser Arbeit waren Aufbau und Validierung einer Verhaltestestbatterie sowie die Implementierung statistischer Prozeduren zur Analyse des kognitiven Langzeitoutcomes in Rattenmodellen der perinatalen Hirnschädigung.

Hypothese: Iatrogene Interventionen (Sedierung) bzw. Umgebungsfaktoren (Hyperoxie) führen zu einer Störung der neuralen Entwicklung und resultieren in einer persistienden kognitiven Beeinträchtigung, welche durch Verhaltenstests nachweisbar sind.

Materialien und Methoden: Zur Validierung der zu etablierenden Tests wurden zwei Tiermodelle verwendet. a) Sechs Tage alte Wistarratten (P6) erhielten, randomisiert, intraperitoneale (i.p.) Injektionen von 3x 30 mg/kg Propofol oder NaCl 0.9% im Abstand von 90 min. b) Fünf tage alte Wistarratten wurden, randomisiert, über 24 h entweder hypoxischen (FiO_2 80%) oder normoxischen Umgebungsbedingungen (FiO_2 21%) ausgesetzt. Generelle Aktivität (Lokomotion, Wegstrecke und Geschwindigkeit), Angst-assoziiertes Verhalten und Habituation wurden mittels "Open field" Test (OF) an vier aufeinanderfolgenden Tagen an zwei Zeitpunkten (P30, P120) beurteilt. Die Lern-/Erinnerungsfähigkeit wurde mittels a) "Novel object recognition" Test bzw. b) duch den "modified Holeboard" Test im Anschluß an den OF Test untersucht.

Ergebnisse: Wir konnten die erforderlichen Routinen und Prozeduren, welche zur Durchführung und statistischen Analyse von Verhaltenstests notwendig sind, erfolgreich etablieren. Propofol behandelte Tiere zeigten eine transiente Steigerung ihrer Aktivität ersten Tag des OF Tests (P30). Wir vermuten, dass diese Hyperaktivität durch die unbekannte Umgebung vervorgerufen wurde. Im Gegensatz zu vorangegangenen Untersuchungen zeigte sich in unserer Studie kein Hinweis auf eine Habituationsstörung der Tiere. Die Untersuchung der Auswirkungen neonataler Hyperoxie, zeigte eine signifikante Veränderung der Lern-/Erinnerungsfähigkeit Hyperoxie behandelter Tiere.

Abstract

Background: Perinatal brain damage is a leading cause of disability and even death in preterm infants, which comprise 5-11% of all live births. Preterm infants are at a high risk of suffering from disturbances in brain development with subsequent neurocognitive sequalae, ranging from cerebral palsy to deficits such as attention deficit and hyperactivity disorder (ADHD). Thus, there is an urgent need to gain insight into the pathophysiology of perinatal brain damage and contributing factors. Currently, important endpoint parameters consist of histological, molecular biological and biochemical analyses to assess neurodegeneration and neuroprotection. It is, however, of utmost importance to also focus on the functional outcome and, therefore, to long-term behavioural effects.

Aim: The aim of this work was to set up a test battery that covers a broad spectrum of behavioural tests to assess long-term cognitive outcome following perinatal brain damage in rodents. This battery includes the routines and protocols for the assessment of long-term cognitive outcome in rodents, as well as the implementation of statistical procedures to analyse the obtained data.

Hypothesis: Environmental factors, a) sedation (propofol) or b) unphysiological oxygen exposure (hyperoxia), disturb neural development, resulting in an impaired cognitive function later in life that can be detected by common behavioural tests.

Materials and Methods: Two animal models were used to evaluate the set-up routines. a) Six-day-old Wistar rats (P6) were randomly assigned to receive either three i.p. injections of 30 mg/kg propofol or NaCl 0.9% solution every 90 min. b) Five-day-old Wistar rats were randomly assigned to 24 h of hyperoxia (FiO_2 80%) or normoxia (FiO_2 21%). Activity (locomotion, travel distance, and speed), anxiety related behaviour and the nature and rate of habituation were assessed by Open Field test (OF) on four consecutive days at two time points (P30, P120). Memory function was assessed by either a) the novel object recognition test or b) the modified hole board test, which subsequently followed the OF.

Results: We have successfully set up several routines and procedures including testing routines and routines for data processing and statistical analyses. In propofol-treated animals, we observed transient increased levels of activity (locomotion and travel distance) on the first day of the open field test (P30). We hypothesise that the hyperactive response of propofol-treated animals was triggered by the novel environment on the first day of observation. In contrast to a previous report, we observed no impairment in the animal's ability to habituate to the testing procedure. We observed a significant difference in memory function in hyperoxia-treated animals, which might indicate an impairment in intermediate to long-term memory in hyperoxia-treated animals.

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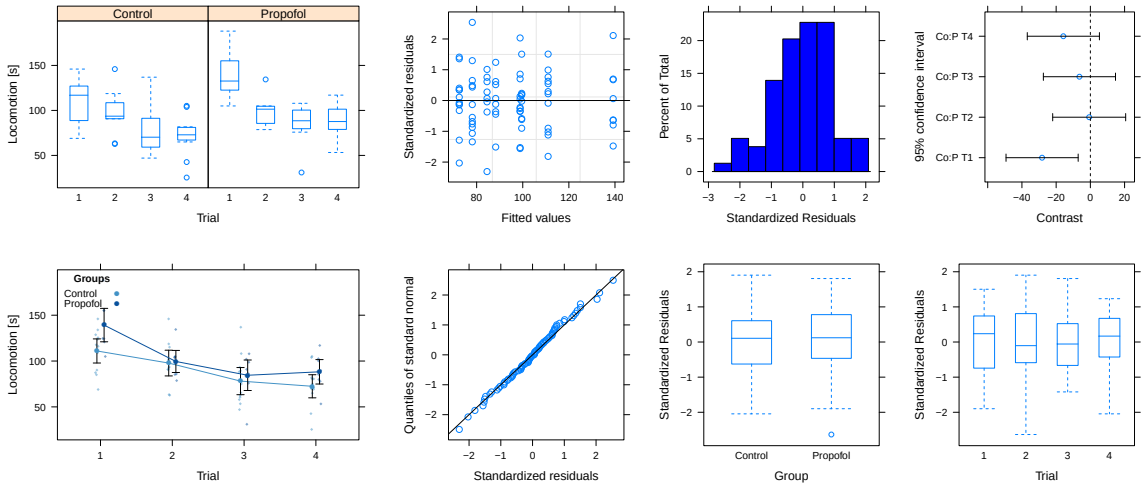
List of Abbreviations

Notation	Description
ADHD	attention deficit hyperactivity disorder
AI	index of anxiety
DI	discrimination index
ELBW	extremely low birth weight (<1000 g)
GABA	γ-Aminobutyric acid
GM	grey matter
mHB	modified hole board test
HI	hypoxia-ischaemia
i.p.	intra-peritoneal
IPA	isopropyl alcohol
IR	infrared
NMDA	N-methyl-D-aspartic acid
NOR	novel object recognition test
OF	open field test
PBS	phosphate-buffered saline
PFA	paraformaldehyde
ROP	retinopathy of prematurity
ROS	reactive oxygen species
RT	room temperature
VLBW	very low birth weight (<1500 g)
WM	white matter

A Supplementary information: Propofol

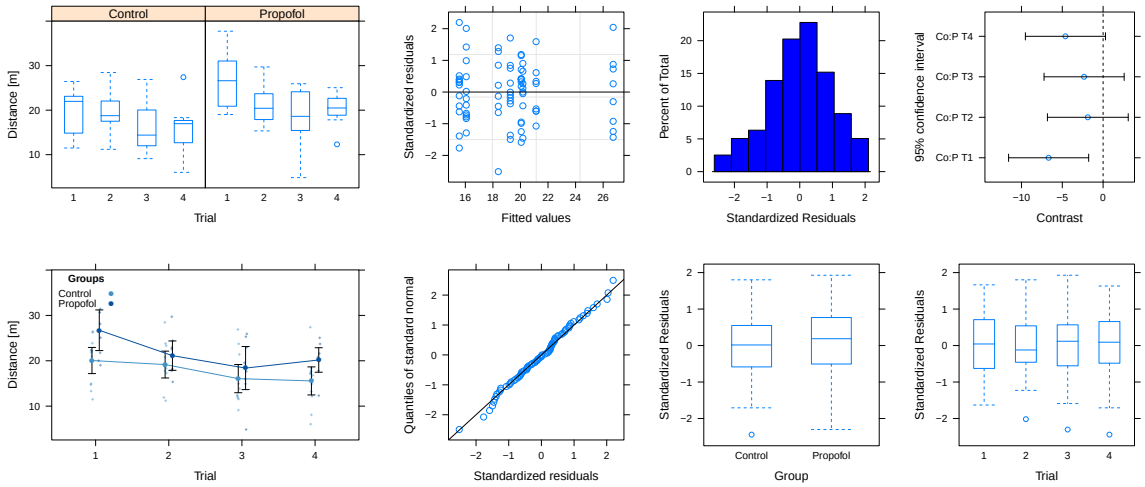
A.1 Open Field (P30)

A.1.1 OF: Lokomotion [s]




```
   Model df      AIC      BIC    logLik   Test   L.Ratio p-value
m      1  9 683.9837 704.3478 -332.9918
m0     2 10 667.4892 690.1160 -323.7446 1 vs 2 18.494433  <.0001
m1     3 11 669.4680 694.3575 -323.7340 2 vs 3  0.021235  0.8841
m2     4 13 672.5568 701.9716 -323.2784 3 vs 4  0.911229  0.6341


Generalized least squares fit by REML
  Model: Lokomotion ~ Trial.f * Gruppe
  Data: of.1.df
       AIC     BIC    logLik
  667.4892 690.116 -323.7446

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
     Rho
0.474907

Coefficients:
                          Value Std.Error   t-value p-value
(Intercept)           111.00050  6.686733 16.600109  0.0000
Trial.f2              -12.15490  7.043478 -1.725696  0.0888
Trial.f3              -32.88842  6.852470 -4.799498  0.0000
Trial.f4              -38.53542  6.852470 -5.623581  0.0000
GruppePropofol         28.21588 10.572653  2.668760  0.0094
Trial.f2:GruppePropofol -27.52610 10.956509 -2.512306  0.0143
Trial.f3:GruppePropofol -21.82583 10.834706 -2.014437  0.0478
Trial.f4:GruppePropofol -12.42471 10.834706 -1.146751  0.2553

Standardized residuals:
       Min         Q1        Med         Q3        Max
-2.30807398 -0.62338854  0.04776468  0.61460048  2.53907474

Residual standard error: 23.16352
Degrees of freedom: 79 total; 71 residual


Denom. DF: 71
             numDF   F-value p-value
(Intercept)      1 275.56362  <.0001
Trial.f          3  13.63048  <.0001
Gruppe           1   7.12228  0.0094
Trial.f:Gruppe   3   2.43036  0.0723


       Contrast S.E. Lower Upper       t df Pr(>|t|)
Co:P T1   -28.22 10.6 -49.3 -7.13 -2.6688 71   0.0377
Co:P T2    -0.69 10.7 -22.0 20.64 -0.0645 71   1.0000
Co:P T3    -6.39 10.6 -27.5 14.69 -0.6044 71   1.0000
Co:P T4   -15.79 10.6 -36.9  5.29 -1.4936 71   0.4191
```

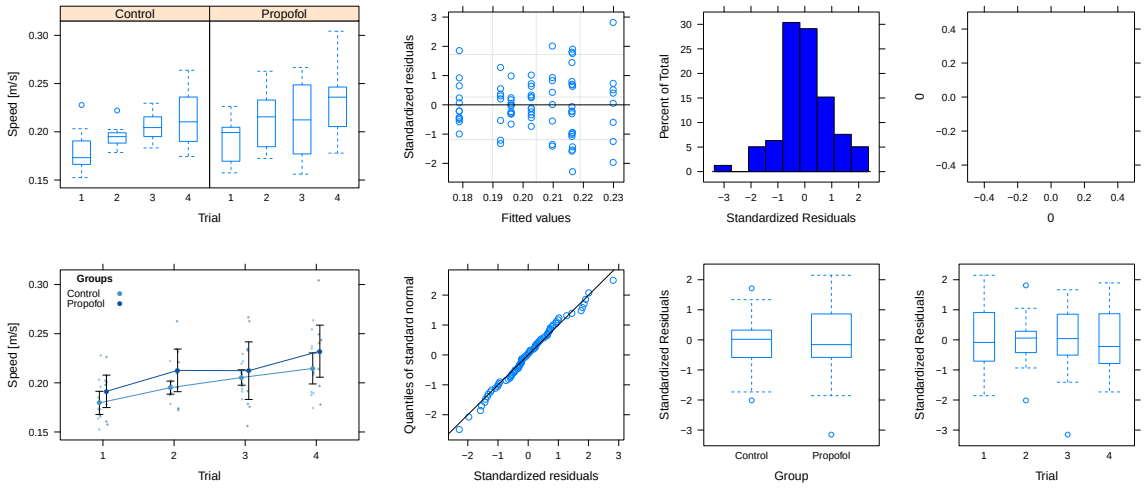
A.1.2 OF: Distance [m]




```
   Model df      AIC      BIC    logLik   Test  L.Ratio p-value
m      1  9 477.3288 497.6929 -229.6644
m0     2 10 446.9493 469.5761 -213.4746 1 vs 2 32.37948  <.0001
m1     3 11 448.8585 473.7480 -213.4292 2 vs 3  0.09079  0.7632
m2     4 13 451.4296 480.8444 -212.7148 3 vs 4  1.42891  0.4895
```

```
Generalized least squares fit by REML
  Model: Globalstrecke ~ Trial.f * Gruppe
  Data: of.1.df
       AIC      BIC    logLik
  446.9493 469.5761 -213.4746

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
      Rho
0.6141865

Coefficients:
                          Value Std.Error   t-value p-value
(Intercept)           20.054608  1.555961 12.888889  0.0000
Trial.f2              -0.791917  1.405859 -0.563298  0.5750
Trial.f3              -3.988992  1.366792 -2.918507  0.0047
Trial.f4              -4.495258  1.366792 -3.288912  0.0016
GruppePropofol         6.673467  2.460190  2.712581  0.0084
Trial.f2:GruppePropofol -4.802408  2.186005 -2.196887  0.0313
Trial.f3:GruppePropofol -4.346921  2.161088 -2.011450  0.0481
Trial.f4:GruppePropofol -2.057904  2.161088 -0.952254  0.3442

Standardized residuals:
        Min          Q1         Med          Q3         Max
-2.51331074 -0.63650311  0.02023807  0.56324602  2.19701940

Residual standard error: 5.390007
Degrees of freedom: 79 total; 71 residual


Denom. DF: 71
              numDF   F-value p-value
(Intercept)       1 166.12345  <.0001
Trial.f           3   5.34795  0.0022
Gruppe            1   7.35810  0.0084
Trial.f:Gruppe    3   2.08607  0.1097


       Contrast S.E.  Lower Upper      t df Pr(>|t|)
Co:P T1    -6.67 2.46 -11.58 -1.77 -2.713 71   0.0335
Co:P T2    -1.87 2.48  -6.82  3.08 -0.754 71   0.6950
Co:P T3    -2.33 2.46  -7.23  2.58 -0.946 71   0.6950
Co:P T4    -4.62 2.46  -9.52  0.29 -1.876 71   0.1943
```

A.1.3 OF: Speed [m/s]



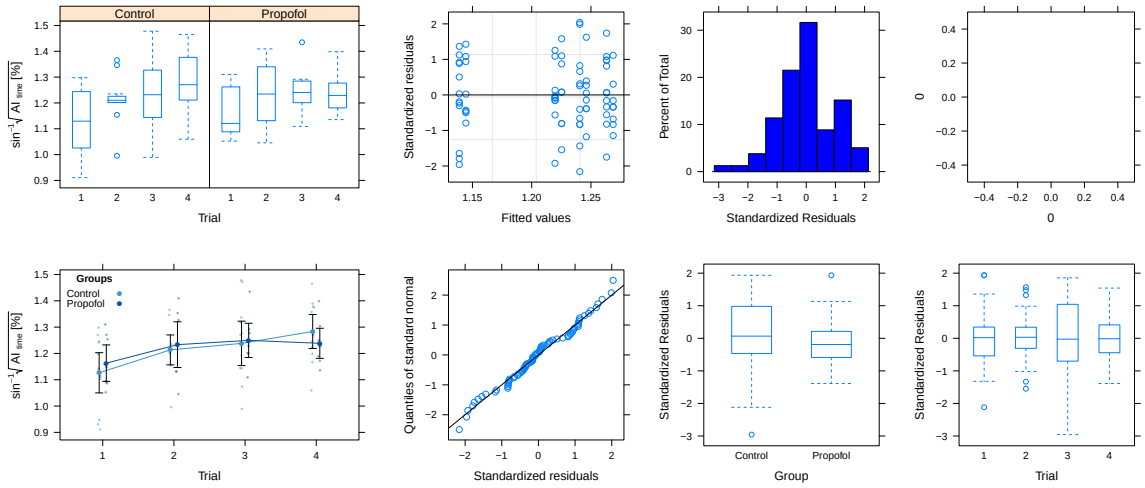

```
   Model df       AIC       BIC   logLik   Test   L.Ratio p-value
m      1  9 -277.3569 -256.9928 147.6785
m0     2 10 -300.5836 -277.9568 160.2918 1 vs 2 25.226678  <.0001
m1     3 11 -304.6257 -279.7362 163.3129 2 vs 3  6.042124  0.0140
m2     4 13 -300.9857 -271.5708 163.4928 3 vs 4  0.359942  0.8353
```

```
Generalized least squares fit by REML
  Model: Geschwindigkeit ~ Trial.f * Gruppe
  Data: of.1.df
       AIC       BIC   logLik
  -300.5836 -277.9568 160.2918

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
    Rho
0.55081

Coefficients:
                              Value   Std.Error   t-value p-value
(Intercept)              0.17960833 0.007671148 23.413489  0.0000
Trial.f2                 0.01424055 0.007476587  1.904686  0.0609
Trial.f3                 0.02581667 0.007270937  3.550666  0.0007
Trial.f4                 0.03505000 0.007270937  4.820562  0.0000
GruppePropofol           0.01172917 0.012129150  0.967023  0.3368
Trial.f2:GruppePropofol  0.00707195 0.011627517  0.608208  0.5450
Trial.f3:GruppePropofol -0.00479167 0.011496361 -0.416799  0.6781
Trial.f4:GruppePropofol  0.00582500 0.011496361  0.506682  0.6139

Standardized residuals:
        Min          Q1         Med          Q3         Max
-2.11723009 -0.55645159 -0.03433855  0.60468769  2.71274515

Residual standard error: 0.02657364
Degrees of freedom: 79 total; 71 residual


Denom. DF: 71
             numDF  F-value p-value
(Intercept)      1 548.1915  <.0001
Trial.f          3   8.6425  0.0001
Gruppe           1   0.9351  0.3368
Trial.f:Gruppe   3   0.4507  0.7176
```

```
        Contrast   S.E.   Lower   Upper      t df Pr(>|t|)
Co:P T1 -0.01173 0.0121 -0.0359 0.01246 -0.967 71    0.674
Co:P T2 -0.01880 0.0123 -0.0432 0.00563 -1.534 71    0.518
Co:P T3 -0.00694 0.0121 -0.0311 0.01725 -0.572 71    0.674
Co:P T4 -0.01755 0.0121 -0.0417 0.00663 -1.447 71    0.518
```

```
Generalized least squares fit by REML
  Model: Geschwindigkeit ~ Trial.f + Gruppe
  Data: of.1.df
       AIC       BIC   logLik
  -327.1949 -311.0664 170.5974

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
     Rho
0.5585951

Coefficients:
                   Value   Std.Error   t-value p-value
(Intercept)    0.17881941 0.007106359 25.163294  0.0000
Trial.f2       0.01719895 0.005642826  3.047931  0.0032
Trial.f3       0.02390000 0.005551103  4.305451  0.0001
Trial.f4       0.03738000 0.005551103  6.733797  0.0000
GruppePropofol 0.01370148 0.009872639  1.387824  0.1694

Standardized residuals:
        Min          Q1         Med          Q3         Max
-2.28300578 -0.54388487 -0.03854232  0.63318611  2.81583388

Residual standard error: 0.0264217
Degrees of freedom: 79 total; 74 residual


Denom. DF: 74
            numDF  F-value p-value
(Intercept)     1 633.1913  <.0001
Trial.f         3  15.6672  <.0001
Gruppe          1   1.9261  0.1694
```

A.1.4 OF: Anxiety [%]




```
   Model df       AIC       BIC   logLik   Test   L.Ratio p-value
m      1  9 -67.09495 -46.73083 42.54748
m0     2 10 -78.28895 -55.66215 49.14447 1 vs 2 13.193996  0.0003
m1     3 11 -79.45711 -54.56763 50.72855 2 vs 3  3.168158  0.0751
m2     4 13 -75.17701 -45.76217 50.58851 3 vs 4  0.280094  0.8693
```

```
Generalized least squares fit by REML
  Model: asin(sqrt(AI.Zeit)) ~ Trial.f * Gruppe
  Data: of.1.df
       AIC       BIC   logLik
  -78.28895 -55.66215 49.14447

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
      Rho
0.3976289

Coefficients:
                              Value  Std.Error  t-value p-value
(Intercept)               1.1264255 0.03369298 33.43205  0.0000
Trial.f2                  0.0872642 0.03799446  2.29676  0.0246
Trial.f3                  0.1118241 0.03698167  3.02377  0.0035
Trial.f4                  0.1569458 0.03698167  4.24388  0.0001
GruppePropofol            0.0366884 0.05327329  0.68868  0.4933
Trial.f2:GruppePropofol -0.0170243 0.05911891 -0.28797  0.7742
Trial.f3:GruppePropofol -0.0253806 0.05847316 -0.43406  0.6656
Trial.f4:GruppePropofol -0.0815525 0.05847316 -1.39470  0.1675

Standardized residuals:
        Min          Q1         Med          Q3         Max
-2.13276625 -0.60617000 -0.07604793  0.78259920  2.05259043

Residual standard error: 0.1167159
Degrees of freedom: 79 total; 71 residual
```

```
Denom. DF: 71
             numDF   F-value p-value
(Intercept)      1 1117.7018  <.0001
Trial.f          3    6.3668  0.0007
Gruppe           1    0.4743  0.4933
Trial.f:Gruppe   3    0.7283  0.5385
```

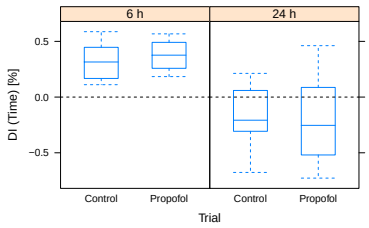
```
        Contrast   S.E.   Lower  Upper      t df Pr(>|t|)
Co:P T1  -0.0367 0.0533 -0.1429 0.0695 -0.689 71        1
Co:P T2  -0.0197 0.0540 -0.1273 0.0880 -0.364 71        1
Co:P T3  -0.0113 0.0533 -0.1175 0.0949 -0.212 71        1
Co:P T4   0.0449 0.0533 -0.0614 0.1511  0.842 71        1
```

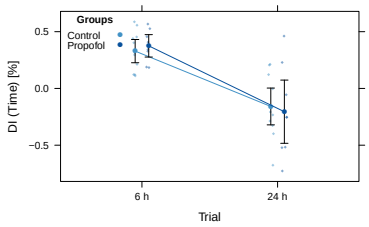
```
Generalized least squares fit by REML
  Model: asin(sqrt(AI.Zeit)) ~ Trial.f + Gruppe
  Data: of.1.df
       AIC       BIC   logLik
  -94.29197 -78.16351 54.14598

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
      Rho
0.4024222

Coefficients:
                 Value  Std.Error  t-value p-value
(Intercept)  1.1388815 0.03043479 37.42039  0.0000
Trial.f2     0.0808160 0.02889262  2.79712  0.0066
Trial.f3     0.1016718 0.02843733  3.57530  0.0006
Trial.f4     0.1243247 0.02843733  4.37189  0.0000
GruppePropofol 0.0055484 0.03950026  0.14046  0.8887

Standardized residuals:
        Min          Q1         Med          Q3         Max
-2.15964599 -0.56100124 -0.08037644  0.84946115  2.03959698

Residual standard error: 0.11633
Degrees of freedom: 79 total; 74 residual
```

```
Denom. DF: 74
             numDF   F-value p-value
(Intercept)      1 1400.2853  <.0001
Trial.f          3    7.2468  0.0003
Gruppe           1    0.0197  0.8887
```

A.2 Novel object recognition (P30)

A.2.1 NOR: Discrimination Time [%]

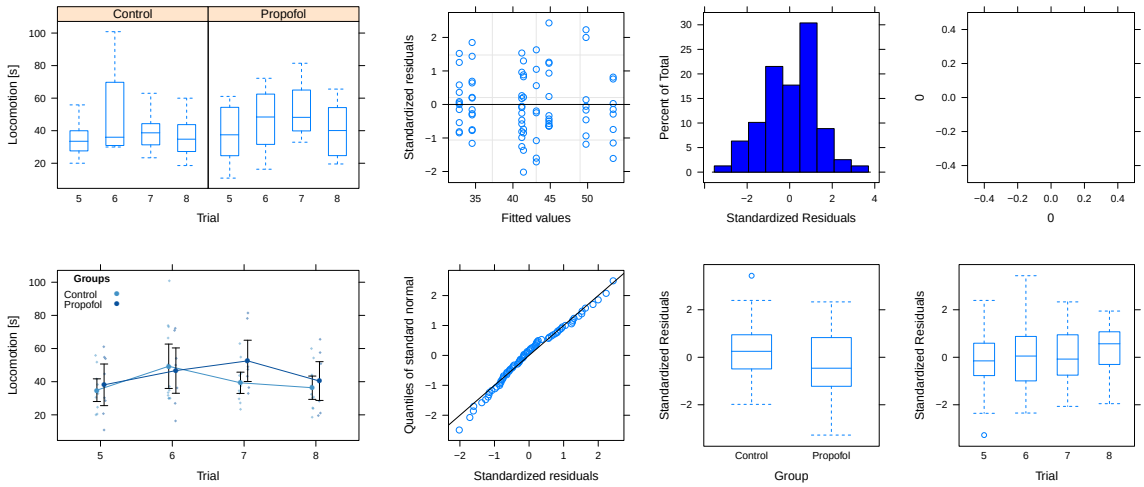





```
        mean  S.E.  Lower  Upper     t df Pr(>|t|)
C 06 h  0.329 0.116  0.212 0.4451  6.30 10 0.000358
P 06 h  0.375 0.119  0.256 0.4943  7.45  7 0.000430
C 24 h -0.159 0.184 -0.343 0.0255 -1.92 10 0.167641
P 24 h -0.205 0.336 -0.541 0.1310 -1.44  7 0.192341
```

A.3 Open Field (P120)

A.3.1 OF: Lokomotion [s]




```
   Model df      AIC      BIC    logLik   Test   L.Ratio p-value
m      1  9 637.7556 658.1197 -309.8778
m0     2 10 613.1985 635.8253 -296.5993 1 vs 2 26.557053  <.0001
m2     3 13 608.4665 637.8814 -291.2333 2 vs 3 10.731990  0.0133
m1     4 11 612.9617 637.8511 -295.4808 3 vs 4  8.495134  0.0143
```

```
Generalized least squares fit by REML
  Model: Lokomotion ~ Trial.f * Gruppe
  Data: of.1.df
       AIC      BIC    logLik
  608.4665 637.8814 -291.2333

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
      Rho
0.6089697
Variance function:
 Structure: Different standard deviations per stratum
 Formula: ~1 | factor(Trial)
 Parameter estimates:
        5         6         7         8
1.0000000 1.5033049 0.9173913 0.9069184

Coefficients:
                            Value Std.Error   t-value p-value
(Intercept)             35.14901  4.474520  7.855370  0.0000
Trial.f6                14.15882  5.313946  2.664465  0.0095
Trial.f7                 4.18474  3.839313  1.089971  0.2794
Trial.f8                 1.21766  3.823439  0.318471  0.7511
GruppePropofol           2.95999  6.982234  0.423932  0.6729
Trial.f6:GruppePropofol -5.57657  8.324262 -0.669918  0.5051
Trial.f7:GruppePropofol 10.26926  5.962305  1.722364  0.0894
Trial.f8:GruppePropofol  1.06097  5.936749  0.178712  0.8587

Standardized residuals:
       Min         Q1        Med         Q3        Max
-1.7972308 -0.7335742 -0.1083741  0.6543210  2.2614340

Residual standard error: 15.16055
Degrees of freedom: 79 total; 71 residual
```

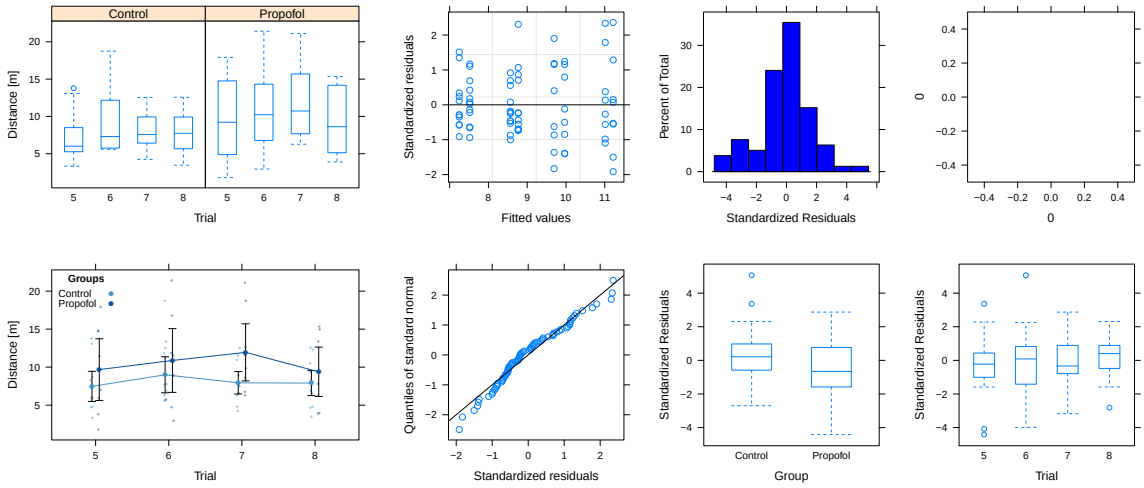
```
Denom. DF: 71
             numDF  F-value p-value
(Intercept)      1 61.70684  <.0001
Trial.f          3  2.65172  0.0552
Gruppe           1  0.17972  0.6729
Trial.f:Gruppe   3  1.79801  0.1554
```

```
        Contrast  S.E. Lower  Upper      t df Pr(>|t|)
Co:P T5    -2.96  6.98 -16.9 10.962 -0.424 71    1.000
Co:P T6     2.62 10.40 -18.1 23.359  0.252 71    1.000
Co:P T7   -13.23  6.35 -25.9 -0.571 -2.084 71    0.163
Co:P T8    -4.02  6.28 -16.5  8.492 -0.641 71    1.000
```

```
Generalized least squares fit by REML
  Model: Lokomotion ~ Trial.f + Gruppe
  Data: of.1.df
       AIC      BIC    logLik
  624.1155 647.1562 -302.0578

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
     Rho
0.599403
Variance function:
 Structure: Different standard deviations per stratum
 Formula: ~1 | factor(Trial)
 Parameter estimates:
        5         6         7         8
1.0000000 1.5218614 0.9384582 0.9092420

Coefficients:
              Value Std.Error  t-value p-value
(Intercept)  32.81878  4.076442 8.050839  0.0000
Trial.f6     12.00527  4.170671 2.878498  0.0052
Trial.f7      8.36952  2.994014 2.795417  0.0066
Trial.f8      1.71912  2.957526 0.581269  0.5628
GruppePropofol  8.59289  5.442619 1.578815  0.1186

Standardized residuals:
       Min         Q1        Med         Q3        Max
-2.0183591 -0.6906478 -0.1500261  0.6679247  2.4321515

Residual standard error: 15.13589
Degrees of freedom: 79 total; 74 residual
```

```
Denom. DF: 74
             numDF  F-value p-value
(Intercept)      1 64.81600  <.0001
Trial.f          3  4.78919  0.0042
Gruppe           1  2.49266  0.1186
```

A.3.2 OF: Distance [m]



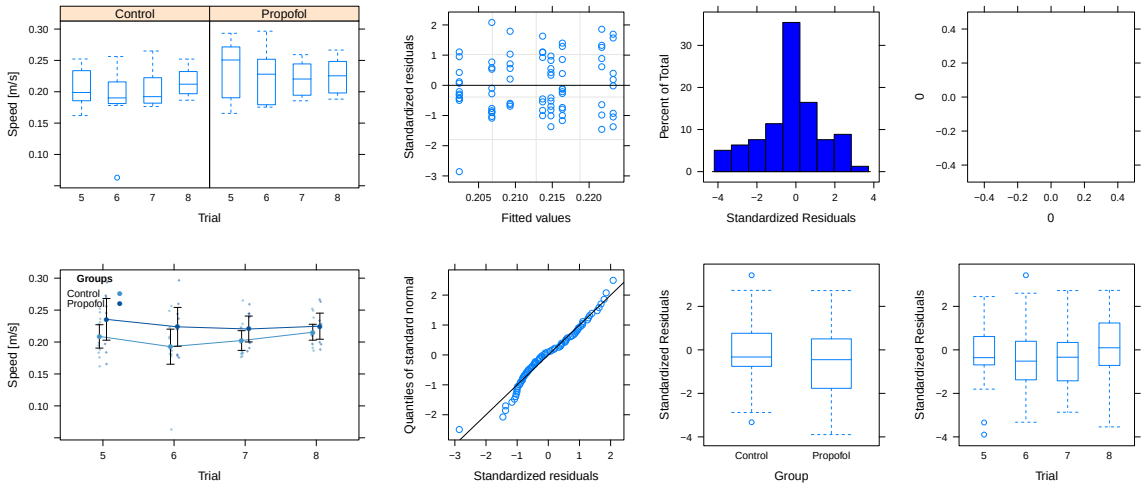

```
   Model df      AIC      BIC    logLik   Test  L.Ratio p-value
m      1  9 445.6072 465.9714 -213.8036
m0     2 10 390.3212 412.9480 -185.1606 1 vs 2 57.28600  <.0001
m1     3 11 391.0246 415.9141 -184.5123 2 vs 3  1.29666  0.2548
m2     4 13 390.6960 420.1109 -182.3480 3 vs 4  4.32856  0.1148
```

```
Generalized least squares fit by REML
  Model: Globalstrecke ~ Trial.f * Gruppe
  Data: of.1.df
       AIC     BIC    logLik
  390.3212 412.948 -185.1606

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
      Rho
0.7713524

Coefficients:
                              Value Std.Error   t-value p-value
(Intercept)                7.258934 1.2619224  5.752282  0.0000
Trial.f6                   1.737625 0.8666112  2.005080  0.0488
Trial.f7                   0.684983 0.8666112  0.790415  0.4319
Trial.f8                   0.644175 0.8666112  0.743326  0.4597
GruppePropofol             2.420866 1.9794225  1.223016  0.2254
Trial.f6:GruppePropofol   -0.539175 1.3470452 -0.400265  0.6902
Trial.f7:GruppePropofol    1.580630 1.3470452  1.173405  0.2446
Trial.f8:GruppePropofol   -0.935362 1.3470452 -0.694381  0.4897

Standardized residuals:
       Min         Q1        Med         Q3        Max
-1.8394933 -0.7202829 -0.2320469  0.6776139  2.4436354

Residual standard error: 4.313389
Degrees of freedom: 79 total; 71 residual


Denom. DF: 71
             numDF  F-value p-value
(Intercept)      1 33.08875  <.0001
Trial.f          3  1.39937  0.2501
Gruppe           1  1.49577  0.2254
Trial.f:Gruppe   3  1.37700  0.2568
```

```
        Contrast S.E. Lower   Upper      t df Pr(>|t|)
Co:P T5    -2.42 1.98 -6.37  1.5260 -1.223 71    0.676
Co:P T6    -1.88 1.97 -5.81  2.0440 -0.956 71    0.685
Co:P T7    -4.00 1.97 -7.93 -0.0759 -2.032 71    0.183
Co:P T8    -1.49 1.97 -5.41  2.4401 -0.755 71    0.685
```

```
Generalized least squares fit by REML
  Model: Globalstrecke ~ Trial.f + Gruppe
  Data: of.1.df
       AIC      BIC    logLik
  395.0136 411.1421 -190.5068

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
      Rho
0.7675474

Coefficients:
                Value Std.Error  t-value p-value
(Intercept)  7.247754 1.2114502 5.982709  0.0000
Trial.f6     1.522444 0.6699865 2.272350  0.0260
Trial.f7     1.317724 0.6699865 1.966792  0.0530
Trial.f8     0.270519 0.6699865 0.403768  0.6875
GruppePropofol 2.447593 1.7927849 1.365247  0.1763

Standardized residuals:
       Min         Q1        Med         Q3        Max
-1.9148808 -0.6287268 -0.2195086  0.6898519  2.3608115

Residual standard error: 4.320891
Degrees of freedom: 79 total; 74 residual


Denom. DF: 74
            numDF  F-value p-value
(Intercept)     1 35.79280  <.0001
Trial.f         3  2.56357  0.0611
Gruppe          1  1.86390  0.1763
```

A.3.3 OF: Speed [m/s]



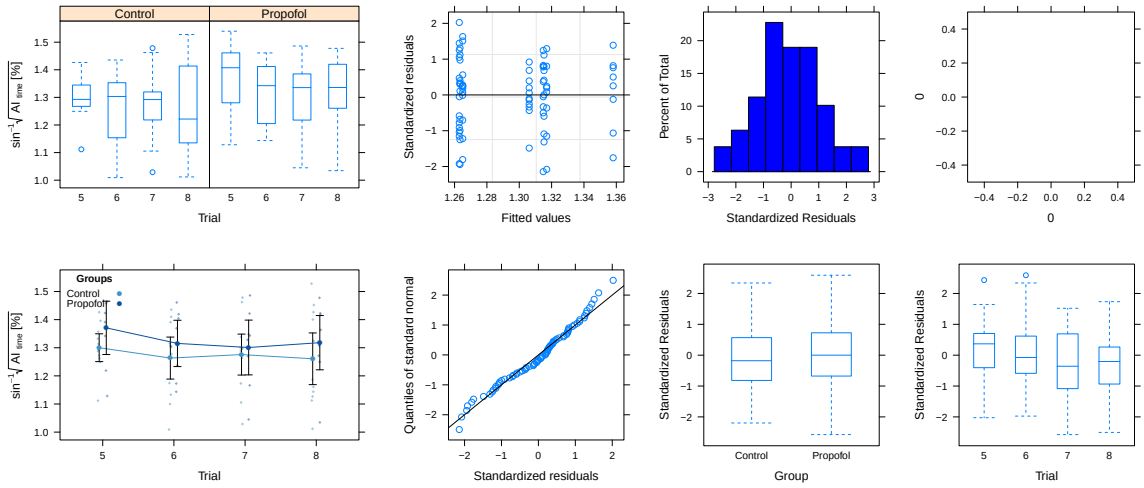

```
   Model df       AIC       BIC   logLik   Test  L.Ratio p-value
m      1  9 -235.9406 -215.5764 126.9703
m0     2 10 -269.4437 -246.8169 144.7218 1 vs 2 35.50311  <.0001
m2     3 13 -280.4135 -250.9986 153.2067 2 vs 3 16.96981   7e-04
m1     4 11 -268.1522 -243.2627 145.0761 3 vs 4 16.26129   3e-04
```

```
Generalized least squares fit by REML
  Model: Geschwindigkeit ~ Trial.f * Gruppe
  Data: of.1.df
       AIC       BIC   logLik
  -280.4135 -250.9987 153.2067

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
      Rho
0.7042636
Variance function:
 Structure: Different standard deviations per stratum
 Formula: ~1 | factor(Trial)
 Parameter estimates:
        5         6         7         8
1.0000000 1.2782766 0.7359199 0.6766051

Coefficients:
                              Value   Std.Error   t-value p-value
(Intercept)                0.20526340 0.011075746 18.532693  0.0000
Trial.f6                  -0.01256340 0.010145616 -1.238308  0.2197
Trial.f7                  -0.00293006 0.007999391 -0.366286  0.7152
Trial.f8                   0.01007827 0.007997633  1.260157  0.2117
GruppePropofol             0.03022410 0.017334977  1.743533  0.0856
Trial.f6:GruppePropofol    0.00087590 0.015847867  0.055269  0.9561
Trial.f7:GruppePropofol   -0.01216994 0.012401479 -0.981329  0.3298
Trial.f8:GruppePropofol   -0.02076577 0.012398646 -1.674842  0.0984

Standardized residuals:
        Min          Q1         Med          Q3         Max
-2.69010068 -0.74850701 -0.08783936  0.74637581  2.25766473

Residual standard error: 0.03771782
Degrees of freedom: 79 total; 71 residual


Denom. DF: 71
             numDF  F-value p-value
(Intercept)      1 343.4607  <.0001
Trial.f          3   2.4701  0.0689
Gruppe           1   3.0399  0.0856
Trial.f:Gruppe   3   1.1217  0.3461
```

```
        Contrast   S.E.   Lower   Upper      t df Pr(>|t|)
Co:P T5 -0.03022 0.0173 -0.0648 0.00434 -1.744 71    0.342
Co:P T6 -0.03110 0.0220 -0.0750 0.01278 -1.413 71    0.476
Co:P T7 -0.01805 0.0127 -0.0433 0.00721 -1.425 71    0.476
Co:P T8 -0.00946 0.0116 -0.0327 0.01377 -0.812 71    0.476
```

```
Generalized least squares fit by REML
  Model: Geschwindigkeit ~ Trial.f + Gruppe
  Data: of.1.df
       AIC       BIC   logLik
  -304.3027 -281.2621 162.1514

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
      Rho
0.7111243
Variance function:
 Structure: Different standard deviations per stratum
 Formula: ~1 | factor(Trial)
 Parameter estimates:
        5         6         7         8
1.0000000 1.2648039 0.7250711 0.6625651

Coefficients:
                    Value   Std.Error   t-value p-value
(Intercept)     0.21481676 0.009631127 22.304426  0.0000
Trial.f6       -0.01243456 0.007809235 -1.592290  0.1156
Trial.f7       -0.00801956 0.006181767 -1.297293  0.1986
Trial.f8        0.00155044 0.006194759  0.250282  0.8031
GruppePropofol  0.00689450 0.010343628  0.666546  0.5071

Standardized residuals:
       Min         Q1        Med         Q3        Max
-2.8583093 -0.7500496 -0.1279005  0.7985749  2.0820368

Residual standard error: 0.03855448
Degrees of freedom: 79 total; 74 residual


Denom. DF: 74
            numDF  F-value p-value
(Intercept)     1 497.4874  <.0001
Trial.f         3   2.2535  0.0892
Gruppe          1   0.4443  0.5071
```

A.3.4 OF: Anxiety [%]




```
   Model df       AIC       BIC   logLik   Test   L.Ratio p-value
m      1  9 -49.83666 -29.47255 33.91833
m0     2 10 -76.24774 -53.62094 48.12387 1 vs 2 28.411074  <.0001
m2     3 13 -73.51038 -44.09554 49.75519 2 vs 3  3.262637  0.3529
m1     4 11 -74.30873 -49.41926 48.15437 3 vs 4  3.201642  0.2017
```

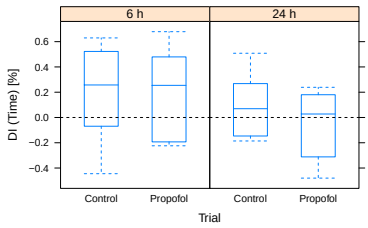
```
Generalized least squares fit by REML
  Model: asin(sqrt(AI.Zeit)) ~ Trial.f * Gruppe
  Data: of.1.df
       AIC       BIC   logLik
  -76.24774 -53.62094 48.12387

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
     Rho
0.579651

Coefficients:
                              Value  Std.Error  t-value p-value
(Intercept)               1.2967310 0.03892738 33.31154  0.0000
Trial.f6                 -0.0334329 0.03584835 -0.93262  0.3542
Trial.f7                 -0.0210049 0.03584835 -0.58594  0.5598
Trial.f8                 -0.0359596 0.03584835 -1.00310  0.3192
GruppePropofol            0.0740228 0.06068995  1.21969  0.2266
Trial.f6:GruppePropofol  -0.0219044 0.05574655 -0.39293  0.6955
Trial.f7:GruppePropofol  -0.0488276 0.05574655 -0.87589  0.3840
Trial.f8:GruppePropofol  -0.0167314 0.05574655 -0.30013  0.7650

Standardized residuals:
       Min         Q1        Med         Q3        Max
-2.1534225 -0.6345178  0.1492306  0.6950257  2.0277192

Residual standard error: 0.1316945
Degrees of freedom: 79 total; 71 residual


Denom. DF: 71
             numDF   F-value p-value
(Intercept)      1 1109.6586  <.0001
Trial.f          3    0.4142  0.7433
Gruppe           1    1.4876  0.2266
Trial.f:Gruppe   3    0.2656  0.8500
```

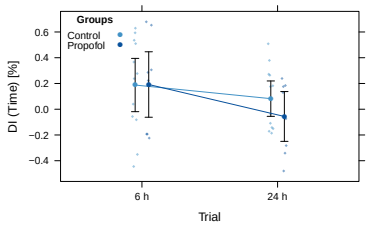
```
        Contrast   S.E.  Lower  Upper      t df Pr(>|t|)
Co:P T5  -0.0740 0.0607 -0.195 0.0470 -1.220 71    0.906
Co:P T6  -0.0521 0.0601 -0.172 0.0677 -0.867 71    1.000
Co:P T7  -0.0252 0.0601 -0.145 0.0947 -0.419 71    1.000
Co:P T8  -0.0573 0.0601 -0.177 0.0626 -0.953 71    1.000
```

```
Generalized least squares fit by REML
  Model: asin(sqrt(AI.Zeit)) ~ Trial.f + Gruppe
  Data: of.1.df
       AIC       BIC   logLik
  -94.03008 -77.90162 54.01504

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
     Rho
0.5906013

Coefficients:
                 Value  Std.Error  t-value p-value
(Intercept)  1.3061409 0.03581925 36.46478  0.0000
Trial.f6    -0.0427634 0.02691009 -1.58912  0.1163
Trial.f7    -0.0411047 0.02691009 -1.52748  0.1309
Trial.f8    -0.0432209 0.02691009 -1.60612  0.1125
GruppePropofol  0.0519200 0.04974803  1.04366  0.3000

Standardized residuals:
       Min         Q1        Med         Q3        Max
-2.1431155 -0.6385879  0.1823200  0.7232996  2.0247875

Residual standard error: 0.130824
Degrees of freedom: 79 total; 74 residual


Denom. DF: 74
            numDF   F-value p-value
(Intercept)     1 1329.6799  <.0001
Trial.f         3    1.2217  0.3079
Gruppe          1    1.0892  0.3000
```

A.4 Novel object recognition (P120)

A.4.1 NOR: Discrimination Time [%]



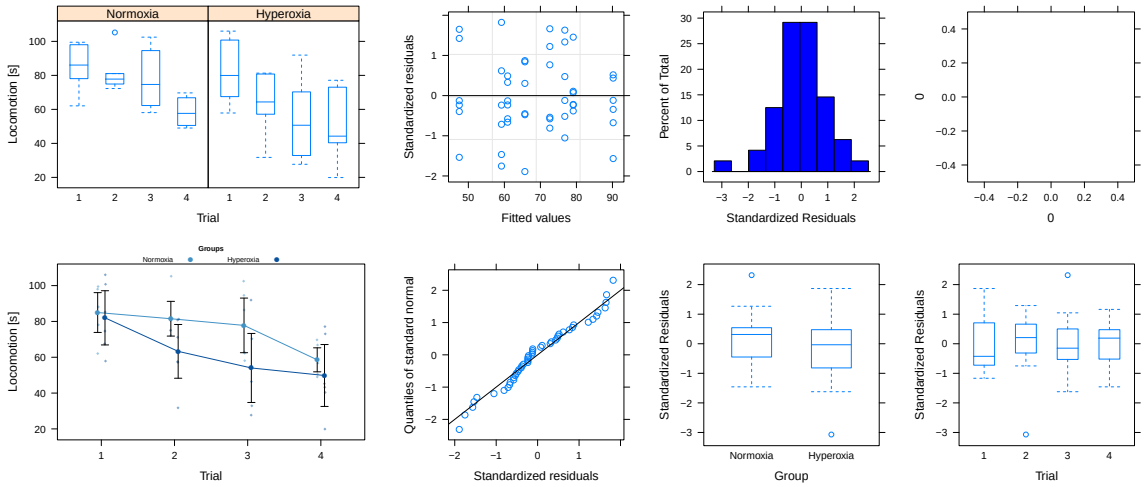


	mean	S.E.	Lower	Upper	t	df	Pr(>|t|)
C 6 h	0.1879	0.232	-0.0445	0.420	1.780	11	0.411
P 6 h	0.1920	0.307	-0.1147	0.499	1.480	7	0.547
C 24 h	0.0821	0.154	-0.0722	0.236	1.171	11	0.547
P 24 h	-0.0562	0.233	-0.2895	0.177	-0.569	7	0.587

B Supplementary information: Hyperoxia

B.1 Open Field Test

B.1.1 OF: Lokomotion [s]



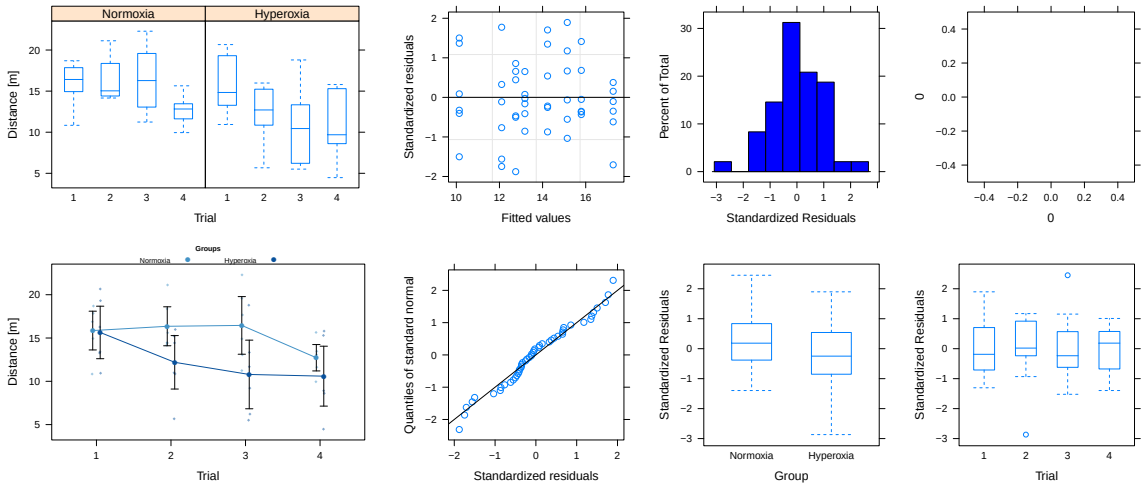

```
   Model df AIC BIC logLik   Test L.Ratio p-value
m      1  9 376 391   -179
m0     2 10 368 385   -174 1 vs 2    9.94  0.0016
m1     3 11 368 387   -173 2 vs 3    1.73  0.1879
m2     4 13 373 395   -173 3 vs 4    0.22  0.8966
```

```
Generalized least squares fit by REML
  Model: Lokomotion ~ Trial.f * Gruppe
  Data: of.1.df
  AIC BIC logLik
  368 385   -174

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.463

Coefficients:
                              Value Std.Error t-value p-value
(Intercept)                    85.0      7.26   11.71  0.0000
Trial.f2                       -3.5      7.52   -0.46  0.6487
Trial.f3                       -7.2      7.52   -0.95  0.3457
Trial.f4                      -26.4      7.52   -3.51  0.0011
GruppeHyperoxia                -3.0     10.26   -0.29  0.7749
Trial.f2:GruppeHyperoxia      -15.3     10.64   -1.44  0.1590
Trial.f3:GruppeHyperoxia      -20.8     10.64   -1.96  0.0574
Trial.f4:GruppeHyperoxia       -5.8     10.64   -0.54  0.5893

Standardized residuals:
    Min      Q1     Med      Q3     Max
-1.7742 -0.5234 -0.0815  0.7554  2.1321

Residual standard error: 17.8
Degrees of freedom: 48 total; 40 residual


Denom. DF: 40
             numDF F-value p-value
(Intercept)      1   137.1  <.0001
Trial.f          3     4.9  0.0053
Gruppe           1     0.1  0.7749
Trial.f:Gruppe   3     1.5  0.2188
```

```
         Contrast S.E.  Lower Upper     t df Pr(>|t|)
NO:HO T1     2.96 10.3 -17.16  23.1 0.288 40    0.799
NO:HO T2    18.22 10.3  -1.90  38.3 1.775 40    0.250
NO:HO T3    23.77 10.3   3.65  43.9 2.316 40    0.103
NO:HO T4     8.74 10.3 -11.37  28.9 0.852 40    0.799
```

```
Generalized least squares fit by REML
  Model: Lokomotion ~ Trial.f + Gruppe
  Data: of.1.df
  AIC BIC logLik
  386 398   -186

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.448

Coefficients:
                Value Std.Error t-value p-value
(Intercept)      90.2      6.53   13.82  0.0000
Trial.f2        -11.1      5.45   -2.03  0.0480
Trial.f3        -17.6      5.45   -3.23  0.0024
Trial.f4        -29.3      5.45   -5.38  0.0000
GruppeHyperoxia -13.4      7.93   -1.69  0.0979

Standardized residuals:
   Min     Q1    Med     Q3    Max
-1.891 -0.549 -0.175  0.542  1.823

Residual standard error: 18
Degrees of freedom: 48 total; 43 residual


Denom. DF: 43
            numDF F-value p-value
(Intercept)     1   191.0  <.0001
Trial.f         3    10.1  <.0001
Gruppe          1     2.9  0.0979
```

B.1.2 OF: Distance [m]



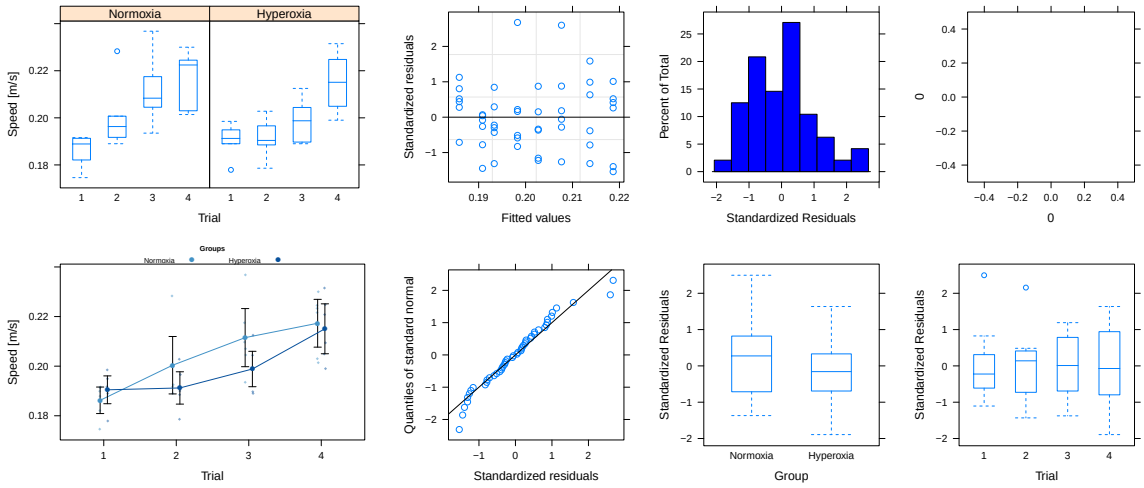

```
   Model df AIC BIC logLik   Test L.Ratio p-value
m      1  9 250 266   -116
m0     2 10 245 262   -112 1 vs 2    7.38  0.0066
m1     3 11 246 265   -112 2 vs 3    0.77  0.3795
m2     4 13 250 271   -112 3 vs 4    0.76  0.6836
```

```
Generalized least squares fit by REML
  Model: Globalstrecke ~ Trial.f * Gruppe
  Data: of.1.df
  AIC BIC logLik
  245 262   -112

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.398

Coefficients:
                            Value Std.Error t-value p-value
(Intercept)                 15.86      1.51   10.52  0.0000
Trial.f2                     0.49      1.65    0.30  0.7667
Trial.f3                     0.59      1.65    0.36  0.7210
Trial.f4                    -3.14      1.65   -1.90  0.0650
GruppeHyperoxia             -0.22      2.13   -0.10  0.9191
Trial.f2:GruppeHyperoxia    -3.94      2.34   -1.69  0.0997
Trial.f3:GruppeHyperoxia    -5.45      2.34   -2.33  0.0250
Trial.f4:GruppeHyperoxia    -1.91      2.34   -0.82  0.4191

Standardized residuals:
    Min      Q1     Med      Q3     Max
-1.7665 -0.5516 -0.0373  0.7087  2.1666

Residual standard error: 3.69
Degrees of freedom: 48 total; 40 residual


Denom. DF: 40
             numDF F-value p-value
(Intercept)      1   110.7  <.0001
Trial.f          3     2.3  0.0929
Gruppe           1     0.0  0.9191
Trial.f:Gruppe   3     2.1  0.1203
```

```
        Contrast S.E.   Lower Upper     t df Pr(>|t|)
NO:HO T1    0.218 2.13 -3.9613  4.40 0.102 40   0.9191
NO:HO T2    4.161 2.13 -0.0183  8.34 1.951 40   0.1741
NO:HO T3    5.665 2.13  1.4854  9.84 2.657 40   0.0451
NO:HO T4    2.128 2.13 -2.0514  6.31 0.998 40   0.6487
```

```
Generalized least squares fit by REML
  Model: Globalstrecke ~ Trial.f + Gruppe
  Data: of.1.df
  AIC BIC logLik
  255 267   -120

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.368

Coefficients:
             Value Std.Error t-value p-value
(Intercept)  17.27      1.34   12.84  0.0000
Trial.f2     -1.48      1.23   -1.21  0.2343
Trial.f3     -2.13      1.23   -1.74  0.0894
Trial.f4     -4.09      1.23   -3.34  0.0017
GruppeHyperoxia -3.04      1.58   -1.93  0.0607

Standardized residuals:
   Min     Q1    Med     Q3    Max
-1.877 -0.476 -0.108  0.654  1.894

Residual standard error: 3.77
Degrees of freedom: 48 total; 43 residual


Denom. DF: 43
             numDF F-value p-value
(Intercept)      1   164.8  <.0001
Trial.f          3     3.8  0.0159
Gruppe           1     3.7  0.0607
```

B.1.3 OF: Speed [m/s]



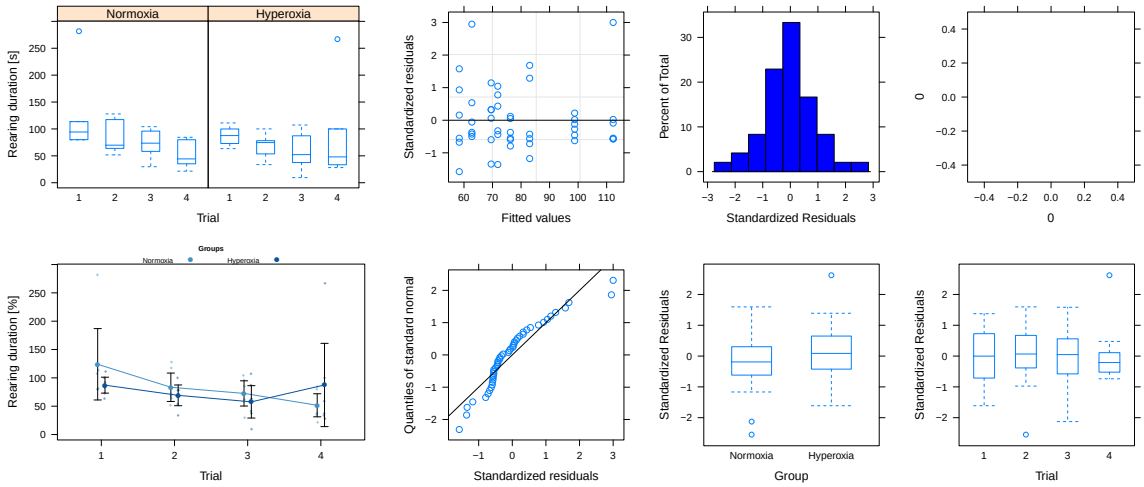

```
   Model df  AIC  BIC logLik   Test L.Ratio p-value
m      1  9 -215 -200    116
m0     2 10 -215 -198    118 1 vs 2    2.04   0.153
m1     3 11 -214 -195    118 2 vs 3    0.51   0.476
m2     4 13 -213 -191    120 3 vs 4    3.60   0.166
```

```
Generalized least squares fit by REML
  Model: Geschwindigkeit ~ Trial.f * Gruppe
  Data: of.1.df
   AIC  BIC logLik
  -215 -198    118

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.202

Coefficients:
                              Value Std.Error t-value p-value
(Intercept)                  0.1862   0.00449    41.5  0.0000
Trial.f2                     0.0142   0.00567     2.5  0.0168
Trial.f3                     0.0253   0.00567     4.5  0.0001
Trial.f4                     0.0311   0.00567     5.5  0.0000
GruppeHyperoxia              0.0042   0.00635     0.7  0.5086
Trial.f2:GruppeHyperoxia    -0.0134   0.00802    -1.7  0.1024
Trial.f3:GruppeHyperoxia    -0.0169   0.00802    -2.1  0.0415
Trial.f4:GruppeHyperoxia    -0.0065   0.00802    -0.8  0.4246

Standardized residuals:
    Min      Q1     Med      Q3     Max
-1.6374 -0.7353 -0.0091  0.5090  2.5395

Residual standard error: 0.011
Degrees of freedom: 48 total; 40 residual


Denom. DF: 40
            numDF F-value p-value
(Intercept)     1    1722  <.0001
Trial.f         3      12  <.0001
Gruppe          1       0   0.509
Trial.f:Gruppe  3       2   0.172
```

```
          Contrast    S.E.     Lower   Upper      t df Pr(>|t|)
NO:HO T1 -0.00423 0.00635 -0.016673 0.00821 -0.667 40    1.000
NO:HO T2  0.00917 0.00635 -0.003273 0.02161  1.444 40    0.469
NO:HO T3  0.01265 0.00635  0.000211 0.02509  1.993 40    0.212
NO:HO T4  0.00223 0.00635 -0.010206 0.01467  0.352 40    1.000
```

```
Generalized least squares fit by REML
  Model: Geschwindigkeit ~ Trial.f + Gruppe
  Data: of.1.df
   AIC  BIC logLik
  -240 -228    127

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.181

Coefficients:
                Value Std.Error t-value p-value
(Intercept)    0.1908   0.00381    50.1  0.0000
Trial.f2       0.0074   0.00414     1.8  0.0791
Trial.f3       0.0168   0.00414     4.1  0.0002
Trial.f4       0.0278   0.00414     6.7  0.0000
GruppeHyperoxia -0.0050   0.00402    -1.2  0.2248

Standardized residuals:
     Min       Q1      Med       Q3      Max
-1.53903 -0.61756 -0.00799  0.51985  2.67700

Residual standard error: 0.0112
Degrees of freedom: 48 total; 43 residual


Denom. DF: 43
          numDF F-value p-value
(Intercept)     1    2507  <.0001
Trial.f         3      17  <.0001
Gruppe          1       2   0.225
```

B.1.4 OF: Rearing [s]



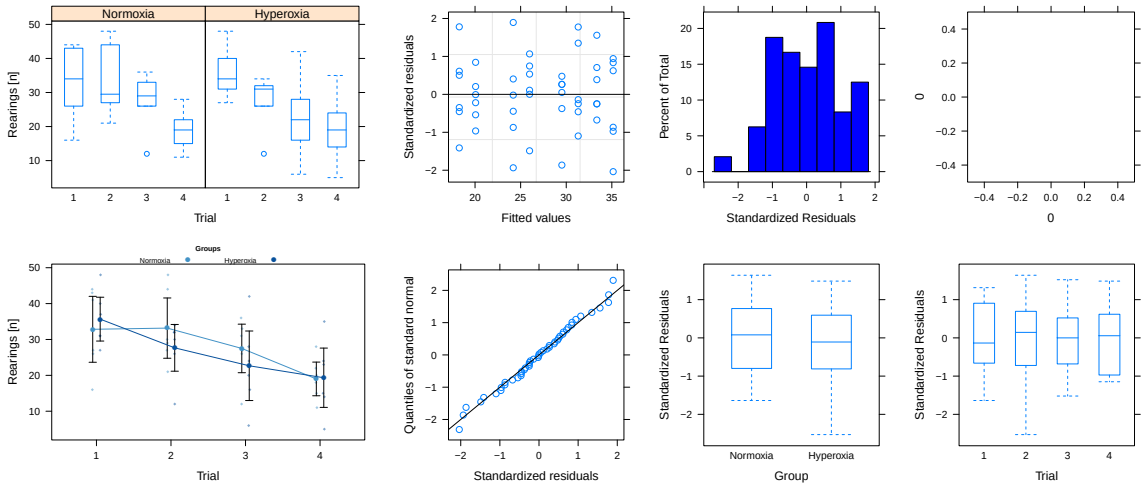

```
   Model df AIC BIC logLik   Test L.Ratio p-value
m      1  9 457 472   -220
m0     2 10 459 476   -220 1 vs 2    0.01  0.9033
m2     3 13 454 476   -214 2 vs 3   10.59  0.0142
m1     4 11 461 479   -219 3 vs 4   10.34  0.0057
```

```
Generalized least squares fit by REML
  Model: Zeit.Rearings ~ Trial.f * Gruppe
  Data: of.1.df
  AIC BIC logLik
  454 476   -214

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
   Rho
0.0192
Variance function:
 Structure: Different standard deviations per stratum
 Formula: ~1 | Trial.f
 Parameter estimates:
    1     2     3     4
1.000 0.476 0.556 1.169

Coefficients:
                          Value Std.Error t-value p-value
(Intercept)               124.0      23.5    5.29  0.0000
Trial.f2                  -40.6      25.8   -1.57  0.1235
Trial.f3                  -51.4      26.6   -1.93  0.0605
Trial.f4                  -72.4      35.7   -2.03  0.0496
GruppeHyperoxia           -36.8      33.2   -1.11  0.2733
Trial.f2:GruppeHyperoxia   22.6      36.5    0.62  0.5389
Trial.f3:GruppeHyperoxia   22.0      37.6    0.58  0.5630
Trial.f4:GruppeHyperoxia   72.7      50.5    1.44  0.1582

Standardized residuals:
   Min     Q1    Med     Q3    Max
-1.507 -0.584 -0.223  0.403  2.746

Residual standard error: 57.5
Degrees of freedom: 48 total; 40 residual


Denom. DF: 40
             numDF F-value p-value
(Intercept)      1   27.93  <.0001
Trial.f          3    1.69   0.185
Gruppe           1    1.23   0.273
Trial.f:Gruppe   3    0.72   0.546
```

```
         Contrast S.E.  Lower Upper      t df Pr(>|t|)
NO:HO T1     36.8 33.2  -28.2 101.9  1.111 40        1
NO:HO T2     14.3 15.8  -16.7  45.2  0.904 40        1
NO:HO T3     14.9 18.5  -21.3  51.1  0.807 40        1
NO:HO T4    -35.8 38.8 -111.8  40.2 -0.924 40        1
```

```
Generalized least squares fit by REML
  Model: Zeit.Rearings ~ Trial.f + Gruppe
  Data: of.1.df
  AIC BIC logLik
  477 495   -228

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
   Rho
0.0212
Variance function:
 Structure: Different standard deviations per stratum
 Formula: ~1 | Trial.f
 Parameter estimates:
    1     2     3     4
1.000 0.472 0.549 1.226

Coefficients:
              Value Std.Error t-value p-value
(Intercept)   112.3      17.2    6.54  0.0000
Trial.f2      -29.3      17.9   -1.64  0.1089
Trial.f3      -40.4      18.4   -2.19  0.0337
Trial.f4      -36.0      25.5   -1.41  0.1653
GruppeHyperoxia -13.5      10.9   -1.24  0.2206

Standardized residuals:
   Min     Q1    Med     Q3    Max
-1.577 -0.567 -0.299  0.324  3.000

Residual standard error: 56.5
Degrees of freedom: 48 total; 43 residual


Denom. DF: 43
             numDF F-value p-value
(Intercept)      1    42.7  <.0001
Trial.f          3     1.6   0.193
Gruppe           1     1.5   0.221
```

B.1.5 OF: Rearing [n]



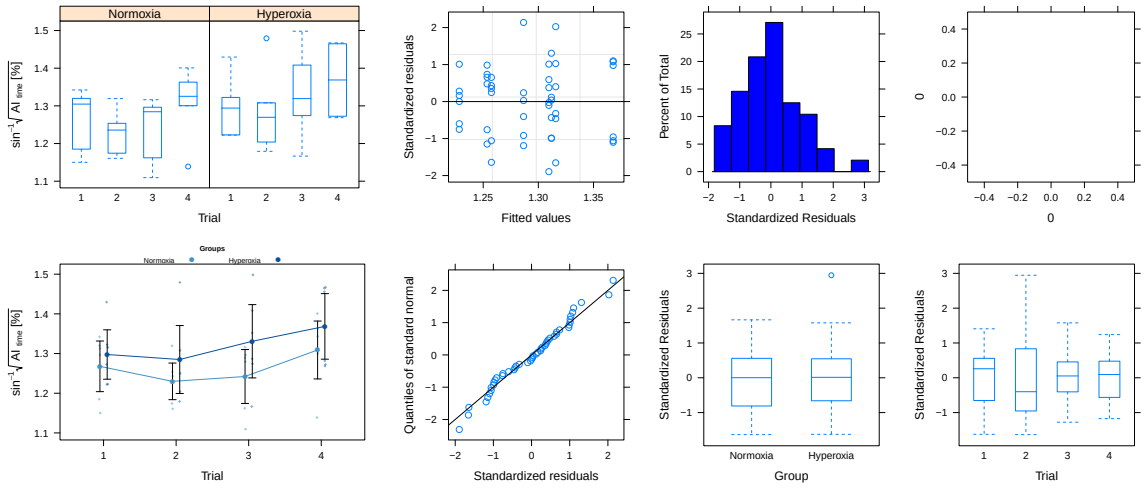

```
   Model df AIC BIC logLik   Test L.Ratio p-value
m      1  9 326 341   -154
m0     2 10 328 345   -154 1 vs 2   0.302   0.583
m1     3 11 330 348   -154 2 vs 3   0.044   0.834
m2     4 13 333 355   -154 3 vs 4   0.438   0.803
```

```
Generalized least squares fit by REML
  Model: Anz.Rearings ~ Trial.f * Gruppe
  Data: of.1.df
  AIC BIC logLik
  328 345   -154

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
   Rho
0.0741

Coefficients:
                          Value Std.Error t-value p-value
(Intercept)                32.8      3.89    8.44  0.0000
Trial.f2                    0.3      5.29    0.06  0.9501
Trial.f3                   -5.3      5.29   -1.01  0.3195
Trial.f4                  -13.8      5.29   -2.61  0.0125
GruppeHyperoxia             2.8      5.50    0.52  0.6092
Trial.f2:GruppeHyperoxia   -8.3      7.48   -1.11  0.2721
Trial.f3:GruppeHyperoxia   -7.7      7.48   -1.02  0.3117
Trial.f4:GruppeHyperoxia   -2.5      7.48   -0.33  0.7401

Standardized residuals:
    Min      Q1     Med      Q3     Max
-1.7674 -0.5731  0.0262  0.5643  2.0299

Residual standard error: 9.52
Degrees of freedom: 48 total; 40 residual


Denom. DF: 40
             numDF F-value p-value
(Intercept)      1    71.3  <.0001
Trial.f          3     3.1  0.0363
Gruppe           1     0.3  0.6092
Trial.f:Gruppe   3     0.6  0.6301
```

```
          Contrast S.E.  Lower Upper       t df Pr(>|t|)
NO:HO T1   -2.833  5.5 -13.61  7.94 -0.5153 40        1
NO:HO T2    5.500  5.5  -5.28 16.28  1.0002 40        1
NO:HO T3    4.833  5.5  -5.94 15.61  0.8790 40        1
NO:HO T4   -0.333  5.5 -11.11 10.44 -0.0606 40        1
```

```
Generalized least squares fit by REML
  Model: Anz.Rearings ~ Trial.f + Gruppe
  Data: of.1.df
  AIC BIC logLik
  340 353   -163

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
   Rho
0.0851

Coefficients:
             Value Std.Error t-value p-value
(Intercept)   35.1      3.11   11.30  0.0000
Trial.f2      -3.8      3.67   -1.04  0.3021
Trial.f3      -9.2      3.67   -2.50  0.0164
Trial.f4     -15.1      3.67   -4.11  0.0002
GruppeHyperoxia  -1.8      3.04   -0.59  0.5587

Standardized residuals:
     Min       Q1      Med       Q3      Max
-2.03725 -0.47883 -0.00222  0.61295  1.89537

Residual standard error: 9.4
Degrees of freedom: 48 total; 43 residual


Denom. DF: 43
             numDF F-value p-value
(Intercept)      1   127.7  <.0001
Trial.f          3     6.4  0.0011
Gruppe           1     0.3  0.5587
```

B.1.6 OF: Anxiety [%]




```
   Model df   AIC   BIC logLik   Test L.Ratio p-value
m      1  9 -45.7 -30.5   31.8
m0     2 10 -51.7 -34.8   35.8 1 vs 2    8.01  0.0046
m1     3 11 -50.0 -31.5   36.0 2 vs 3    0.37  0.5420
m2     4 13 -45.8 -23.8   35.9 3 vs 4    0.28  0.8707
```

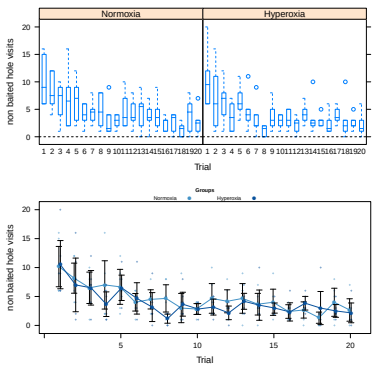
```
Generalized least squares fit by REML
  Model: asin(sqrt(AI.Zeit)) ~ Trial.f * Gruppe
  Data: of.1.df
    AIC   BIC logLik
  -51.7 -34.8   35.8

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.415

Coefficients:
                            Value Std.Error t-value p-value
(Intercept)                 1.268    0.0373    34.0   0.000
Trial.f2                   -0.038    0.0403    -0.9   0.353
Trial.f3                   -0.026    0.0403    -0.6   0.529
Trial.f4                    0.041    0.0403     1.0   0.313
GruppeHyperoxia             0.030    0.0527     0.6   0.575
Trial.f2:GruppeHyperoxia    0.025    0.0570     0.4   0.660
Trial.f3:GruppeHyperoxia    0.059    0.0570     1.0   0.307
Trial.f4:GruppeHyperoxia    0.030    0.0570     0.5   0.606

Standardized residuals:
   Min     Q1    Med     Q3    Max
-1.863 -0.820  0.170  0.593  2.128

Residual standard error: 0.0913
Degrees of freedom: 48 total; 40 residual


Denom. DF: 40
             numDF F-value p-value
(Intercept)      1    1157  <.0001
Trial.f          3       2   0.228
Gruppe           1       0   0.575
Trial.f:Gruppe   3       0   0.782
```

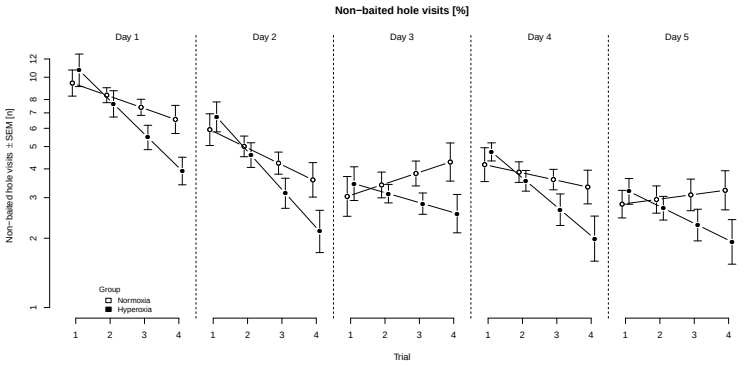
```
         Contrast   S.E.  Lower  Upper      t df Pr(>|t|)
NO:HO T1  -0.0298 0.0527 -0.133 0.0735 -0.565 40    0.798
NO:HO T2  -0.0551 0.0527 -0.158 0.0482 -1.045 40    0.798
NO:HO T3  -0.0888 0.0527 -0.192 0.0145 -1.685 40    0.399
NO:HO T4  -0.0594 0.0527 -0.163 0.0439 -1.128 40    0.798
```

```
Generalized least squares fit by REML
  Model: asin(sqrt(AI.Zeit)) ~ Trial.f + Gruppe
  Data: of.1.df
  AIC   BIC logLik
  -69 -56.7   41.5

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.435

Coefficients:
                Value Std.Error t-value p-value
(Intercept)     1.254    0.0327    38.4  0.0000
Trial.f2       -0.025    0.0277    -0.9  0.3668
Trial.f3        0.004    0.0277     0.1  0.8884
Trial.f4        0.056    0.0277     2.0  0.0491
GruppeHyperoxia 0.058    0.0395     1.5  0.1473

Standardized residuals:
    Min      Q1     Med      Q3     Max
-1.8935 -0.7988  0.0792  0.6538  2.1383

Residual standard error: 0.0901
Degrees of freedom: 48 total; 43 residual


Denom. DF: 43
            numDF F-value p-value
(Intercept)     1    1473  <.0001
Trial.f         3       3   0.039
Gruppe          1       2   0.147
```

B.2 modified Holeboard Test

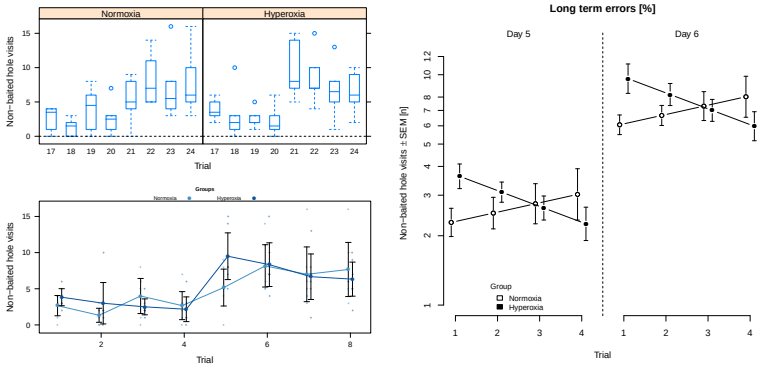
B.2.1 mHB: non-baited hole visits (Day 1–5) [n]






```
Call:
geeglm(formula = no.bait ~ Day.f * Trial.rep * Gruppe, family = poisson,
    data = mHB.KY.d15, na.action = na.omit, id = Tier.Nr., waves = Trial,
    corstr = "exchangeable")

 Coefficients:
                                Estimate Std.err  Wald Pr(>|W|)
(Intercept)                       2.4146  0.2780 75.46  < 2e-16 ***
Day.f2                           -0.5430  0.4954  1.20   0.2731
Day.f3                           -1.4945  0.5159  8.39   0.0038 **
Day.f4                           -0.7021  0.2148 10.68   0.0011 **
Day.f5                           -1.6907  0.4226 16.01  6.3e-05 ***
Trial.rep                        -0.1411  0.1046  1.82   0.1772
GruppeHyperoxia                   0.2394  0.3626  0.44   0.5092
Day.f2:Trial.rep                  0.0298  0.1585  0.04   0.8509
Day.f3:Trial.rep                  0.2874  0.1817  2.50   0.1136
Day.f4:Trial.rep                 -0.0268  0.1083  0.06   0.8049
Day.f5:Trial.rep                  0.2414  0.1658  2.12   0.1455
Day.f2:GruppeHyperoxia            0.3052  0.5498  0.31   0.5788
Day.f3:GruppeHyperoxia            0.2672  0.5949  0.20   0.6533
Day.f4:GruppeHyperoxia           -0.3284  0.4195  0.61   0.4337
Day.f5:GruppeHyperoxia            0.5533  0.4903  1.27   0.2591
Trial.rep:GruppeHyperoxia        -0.1707  0.1146  2.22   0.1363
Day.f2:Trial.rep:GruppeHyperoxia -0.1983  0.2172  0.83   0.3611
Day.f3:Trial.rep:GruppeHyperoxia -0.1172  0.2128  0.30   0.5817
Day.f4:Trial.rep:GruppeHyperoxia  0.1522  0.1711  0.79   0.3735
Day.f5:Trial.rep:GruppeHyperoxia -0.1228  0.1790  0.47   0.4926
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Estimated Scale Parameters:
            Estimate Std.err
(Intercept)     1.62   0.149

Correlation: Structure = exchangeable  Link = identity

Estimated Correlation Parameters:
      Estimate Std.err
alpha   0.0243  0.0272
Number of clusters:   12   Maximum cluster size: 20


Analysis of 'Wald statistic' Table
Model: poisson, link: log
Response: no.bait
Terms added sequentially (first to last)

                      Df   X2 P(>|Chi|)
Day.f                  4 79.8   2.2e-16 ***
Trial.rep              1 10.4    0.0012 **
Gruppe                 1  1.7    0.1874
Day.f:Trial.rep        4 16.3    0.0026 **
Day.f:Gruppe           4  1.4    0.8432
Trial.rep:Gruppe       1  8.2    0.0043 **
Day.f:Trial.rep:Gruppe 4  3.5    0.4781
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


Call:
geeglm(formula = no.bait ~ Day.f + Trial.rep + Gruppe + Day.f:Trial.rep +
    Trial.rep:Gruppe, family = poisson, data = mHB.KY.d15, na.action = na.omit,
    id = Tier.Nr., waves = Trial, corstr = "exchangeable")

 Coefficients:
                             Estimate Std.err   Wald Pr(>|W|)
(Intercept)                   2.3652  0.2255 110.05  < 2e-16 ***
Day.f2                       -0.4195  0.2590   2.62  0.10522
Day.f3                       -1.3686  0.2922  21.94  2.8e-06 ***
Day.f4                       -0.8621  0.2275  14.36  0.00015 ***
Day.f5                       -1.3775  0.2514  30.02  4.3e-08 ***
Trial.rep                    -0.1212  0.0843   2.07  0.15032
GruppeHyperoxia               0.3419  0.2344   2.13  0.14472
Day.f2:Trial.rep             -0.0461  0.1013   0.21  0.64882
Day.f3:Trial.rep              0.2357  0.1070   4.85  0.02760 *
Day.f4:Trial.rep              0.0466  0.0931   0.25  0.61637
Day.f5:Trial.rep              0.1673  0.0959   3.04  0.08117 .
Trial.rep:GruppeHyperoxia    -0.2144  0.0811   6.98  0.00824 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Estimated Scale Parameters:
            Estimate Std.err
(Intercept)     1.64   0.164

Correlation: Structure = exchangeable  Link = identity

Estimated Correlation Parameters:
      Estimate Std.err
alpha   0.0221  0.0281
Number of clusters:   12   Maximum cluster size: 20


Analysis of 'Wald statistic' Table
Model: poisson, link: log
Response: no.bait
Terms added sequentially (first to last)

                    Df   X2 P(>|Chi|)
Day.f                4 79.8   2.2e-16 ***
Trial.rep            1 10.4    0.0012 **
Gruppe               1  1.7    0.1874
Day.f:Trial.rep      4 16.3    0.0026 **
Trial.rep:Gruppe     1  7.0    0.0082 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


            Estimate Std.Error X2.value DF Pr(>|X^2|)    Lower    Upper
NO D1 T1:T4   -0.3636     0.253  2.06899  1   0.601286 -0.8590   0.1318
NO D2 T1:T4   -0.5020     0.307  2.67438  1   0.509876 -1.1036   0.0996
NO D3 T1:T4    0.3436     0.358  0.91954  1   1.000000 -0.3587   1.0460
NO D4 T1:T4   -0.2237     0.316  0.50240  1   1.000000 -0.8422   0.3948
NO D5 T1:T4    0.1383     0.181  0.58671  1   1.000000 -0.2156   0.4921
HO D1 T1:T4   -1.0067     0.164 37.59076  1   0.000000 -1.3286 -0.6849
HO D2 T1:T4   -1.1451     0.274 17.44949  1   0.000118 -1.6824 -0.6078
HO D3 T1:T4   -0.2995     0.338  0.78496  1   0.375628 -0.9621   0.3631
HO D4 T1:T4   -0.8668     0.227 14.55574  1   0.000408 -1.3121 -0.4215
HO D5 T1:T4   -0.5049     0.266  3.59279  1   0.116062 -1.0269   0.0172
NO D1T4:D2T1  -0.1021     0.264  0.14934  1   1.000000 -0.6198   0.4156
NO D2T4:D3T1  -0.1653     0.303  0.29820  1   1.000000 -0.7584   0.4279
NO D3T4:D4T1  -0.0262     0.338  0.00602  1   1.000000 -0.6889   0.6364
NO D4T4:D5T1  -0.1710     0.235  0.52763  1   1.000000 -0.6324   0.2904
HO D1T4:D2T1   0.5411     0.162 11.11943  1   0.003417  0.2230   0.8591
HO D2T4:D3T1   0.4779     0.319  2.24577  1   0.133980 -0.1471   1.1029
HO D3T4:D4T1   0.6169     0.207  8.87053  1   0.008694  0.2109   1.0229
HO D4T4:D5T1   0.4721     0.211  4.99451  1   0.050856  0.0581   0.8862
```

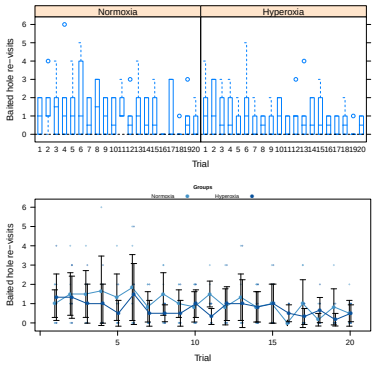
B.2.2 mHB: non-baited hole visits (Day 5–6) [n]

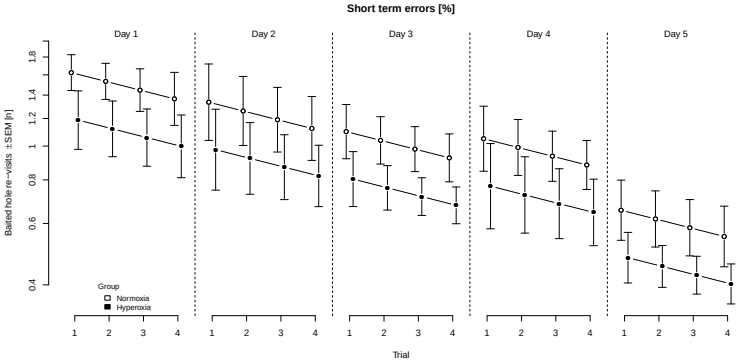



```
Call:
geeglm(formula = no.bait ~ Day.f * Trial.rep * Gruppe, family = poisson,
    data = mHB.KY.d56, na.action = na.omit, id = Tier.Nr., waves = Trial,
    corstr = "exchangeable")

 Coefficients:
                                 Estimate Std.err  Wald Pr(>|W|)
(Intercept)                        0.7239  0.1677 18.63  1.6e-05 ***
Day.f6                             0.9902  0.1640 36.44  1.6e-09 ***
Trial.rep                          0.1003  0.0983  1.04  0.30761
GruppeHyperoxia                    0.7921  0.2246 12.43  0.00042 ***
Day.f6:Trial.rep                  -0.0096  0.0491  0.04  0.84513
Day.f6:GruppeHyperoxia            -0.1129  0.3311  0.12  0.73322
Trial.rep:GruppeHyperoxia         -0.2936  0.1154  6.47  0.01095 *
Day.f6:Trial.rep:GruppeHyperoxia   0.0572  0.1064  0.29  0.59086
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Estimated Scale Parameters:
            Estimate Std.err
(Intercept)     1.74   0.144

Correlation: Structure = exchangeable  Link = identity

Estimated Correlation Parameters:
      Estimate Std.err
alpha    0.137   0.101
Number of clusters:   12   Maximum cluster size: 8


Analysis of 'Wald statistic' Table
Model: poisson, link: log
Response: no.bait
Terms added sequentially (first to last)

                     Df   X2 P(>|Chi|)
Day.f                 1 92.9    <2e-16 ***
Trial.rep             1  0.3     0.583
Gruppe                1  0.3     0.586
Day.f:Trial.rep       1  0.1     0.768
Day.f:Gruppe          1  0.0     0.931
Trial.rep:Gruppe      1  5.3     0.021 *
Day.f:Trial.rep:Gruppe  1  0.3     0.591
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Call:
geeglm(formula = no.bait ~ Day.f + Trial.rep + Gruppe + Trial.rep:Gruppe,
    family = poisson, data = mHB.KY.d56, na.action = na.omit,
    id = Tier.Nr., waves = Trial, corstr = "exchangeable")

 Coefficients:
                           Estimate Std.err  Wald Pr(>|W|)
(Intercept)                  0.7329  0.1714 18.28  1.9e-05 ***
Day.f6                       0.9759  0.1009 93.62  < 2e-16 ***
Trial.rep                    0.0934  0.0851  1.20    0.272
GruppeHyperoxia              0.7129  0.2474  8.30    0.004 **
Trial.rep:GruppeHyperoxia   -0.2519  0.1086  5.37    0.020 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Estimated Scale Parameters:
            Estimate Std.err
(Intercept)     1.74   0.145

Correlation: Structure = exchangeable  Link = identity

Estimated Correlation Parameters:
      Estimate Std.err
alpha    0.138  0.0982
Number of clusters:   12   Maximum cluster size: 8


Analysis of 'Wald statistic' Table
Model: poisson, link: log
Response: no.bait
Terms added sequentially (first to last)

                Df   X2 P(>|Chi|)
Day.f            1 92.9    <2e-16 ***
Trial.rep        1  0.3      0.58
Gruppe           1  0.3      0.59
Trial.rep:Gruppe  1  5.4      0.02 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


          Estimate Std.Error X2.value DF Pr(>|X^2|)   Lower   Upper
NO T1:T4     0.280     0.255     1.20  1     0.2724 -0.2201  0.7805
HO T1:T4    -0.475     0.203     5.46  1     0.0194 -0.8740 -0.0768
NO D5T4:D6T1 0.696     0.307     5.14  1     0.0233  0.0945  1.2971
HO D5T4:D6T1 1.451     0.257    31.96  1     0.0000  0.9482  1.9545
```

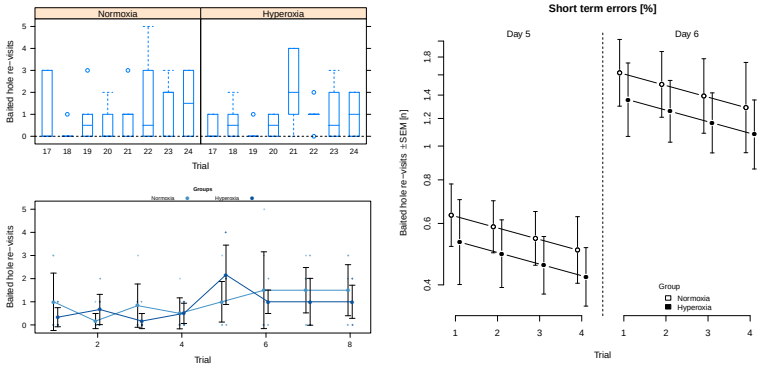
B.2.3 mHB: baited hole re-visits (Day 1–5) [n]





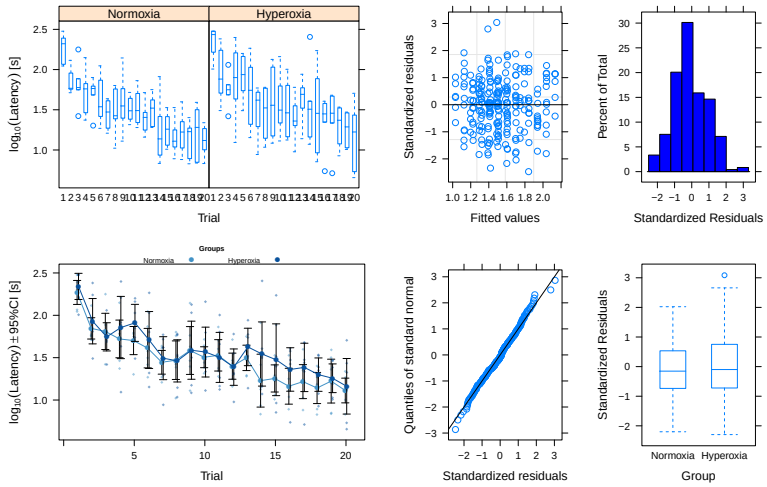

```
Call:
geeglm(formula = re.bait ~ Day.f * Trial.rep * Gruppe, family = poisson,
    data = mHB.KY.d15, na.action = na.omit, id = Tier.Nr., waves = Trial,
    corstr = "exchangeable")

 Coefficients:
                                 Estimate Std.err Wald Pr(>|W|)
(Intercept)                       -0.0200  0.3902 0.00    0.959
Day.f2                             0.4279  1.0875 0.15    0.694
Day.f3                             0.0199  0.6312 0.00    0.975
Day.f4                             0.8584  0.6156 1.94    0.163
Day.f5                            -0.1270  1.2412 0.01    0.918
Trial.rep                          0.1420  0.1279 1.23    0.267
GruppeHyperoxia                    0.4527  0.6438 0.49    0.482
Day.f2:Trial.rep                  -0.1784  0.3253 0.30    0.583
Day.f3:Trial.rep                  -0.1260  0.2387 0.28    0.598
Day.f4:Trial.rep                  -0.6168  0.2767 4.97    0.026 *
Day.f5:Trial.rep                  -0.2761  0.4438 0.39    0.534
Day.f2:GruppeHyperoxia            -0.8245  1.2283 0.45    0.502
Day.f3:GruppeHyperoxia            -1.1015  0.8089 1.85    0.173
Day.f4:GruppeHyperoxia            -1.0866  0.7165 2.30    0.129
Day.f5:GruppeHyperoxia            -1.1813  1.5957 0.55    0.459
Trial.rep:GruppeHyperoxia         -0.2567  0.2485 1.07    0.302
Day.f2:Trial.rep:GruppeHyperoxia   0.1591  0.4062 0.15    0.695
Day.f3:Trial.rep:GruppeHyperoxia   0.3589  0.3291 1.19    0.275
Day.f4:Trial.rep:GruppeHyperoxia   0.5703  0.3337 2.92    0.087 .
Day.f5:Trial.rep:GruppeHyperoxia   0.3908  0.5709 0.47    0.494
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Estimated Scale Parameters:
            Estimate Std.err
(Intercept)     1.29    0.13

Correlation: Structure = exchangeable  Link = identity

Estimated Correlation Parameters:
      Estimate Std.err
alpha  -0.0316    0.02
Number of clusters:   12   Maximum cluster size: 20


Analysis of 'Wald statistic' Table
Model: poisson, link: log
Response: re.bait
Terms added sequentially (first to last)

                      Df    X2 P(>|Chi|)
Day.f                  4 20.59   0.00038 ***
Trial.rep              1  1.29   0.25535
Gruppe                 1  8.39   0.00378 **
Day.f:Trial.rep        4  4.99   0.28818
Day.f:Gruppe           4  1.67   0.79651
Trial.rep:Gruppe       1  0.01   0.92181
Day.f:Trial.rep:Gruppe 4  3.74   0.44223
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


Call:
geeglm(formula = re.bait ~ Day.f + Trial.rep + Gruppe, family = poisson,
    data = mHB.KY.d15, na.action = na.omit, id = Tier.Nr., waves = Trial,
    corstr = "exchangeable")

 Coefficients:
             Estimate Std.err  Wald Pr(>|W|)
(Intercept)    0.5427  0.1503 13.04  0.00031 ***
Day.f2        -0.1953  0.2818  0.48  0.48827
Day.f3        -0.3895  0.1789  4.74  0.02949 *
Day.f4        -0.4363  0.2489  3.07  0.07963 .
Day.f5        -0.9083  0.2584 12.36  0.00044 ***
Trial.rep     -0.0578  0.0536  1.16  0.28144
GruppeHyperoxia -0.3134  0.1082  8.39  0.00378 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Estimated Scale Parameters:
            Estimate Std.err
(Intercept)     1.34   0.125

Correlation: Structure = exchangeable  Link = identity

Estimated Correlation Parameters:
      Estimate Std.err
alpha  -0.0318  0.0165
Number of clusters:   12   Maximum cluster size: 20


Analysis of 'Wald statistic' Table
Model: poisson, link: log
Response: re.bait
Terms added sequentially (first to last)

          Df    X2 P(>|Chi|)
Day.f      4 20.59   0.00038 ***
Trial.rep  1  1.29   0.25535
Gruppe     1  8.39   0.00378 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

B.2.4 mHB: baited hole re-visits (Day 5–6) [n]



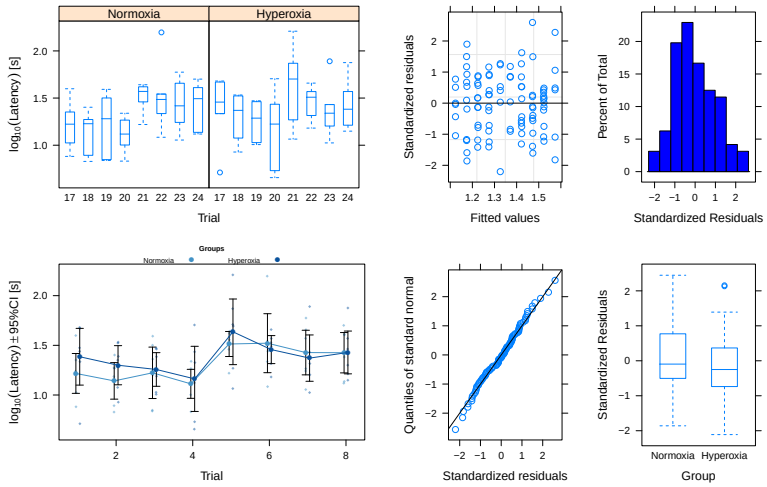

```
Call:
geeglm(formula = re.bait ~ Day.f * Trial.rep * Gruppe, family = poisson,
    data = mHB.KY.d56, na.action = na.omit, id = Tier.Nr., waves = Trial,
    corstr = "exchangeable")

 Coefficients:
                                 Estimate Std.err Wald Pr(>|W|)
(Intercept)                        -0.146   1.011 0.02     0.89
Day.f6                              0.183   1.149 0.03     0.87
Trial.rep                          -0.134   0.366 0.13     0.71
GruppeHyperoxia                    -0.729   1.138 0.41     0.52
Day.f6:Trial.rep                    0.243   0.348 0.49     0.48
Day.f6:GruppeHyperoxia              1.592   1.395 1.30     0.25
Trial.rep:GruppeHyperoxia           0.134   0.399 0.11     0.74
Day.f6:Trial.rep:GruppeHyperoxia   -0.520   0.439 1.40     0.24

Estimated Scale Parameters:
            Estimate Std.err
(Intercept)     1.18   0.161

Correlation: Structure = exchangeable  Link = identity

Estimated Correlation Parameters:
      Estimate Std.err
alpha  -0.0563  0.0292
Number of clusters:   12   Maximum cluster size: 8



Analysis of 'Wald statistic' Table
Model: poisson, link: log
Response: re.bait
Terms added sequentially (first to last)

                     Df   X2 P(>|Chi|)
Day.f                 1 8.47    0.0036 **
Trial.rep             1 0.68    0.4088
Gruppe                1 0.83    0.3631
Day.f:Trial.rep       1 0.00    0.9831
Day.f:Gruppe          1 0.30    0.5813
Trial.rep:Gruppe      1 2.66    0.1029
Day.f:Trial.rep:Gruppe 1 1.40    0.2360
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1


Call:
geeglm(formula = re.bait ~ Day.f + Trial.rep + Gruppe, family = poisson,
    data = mHB.KY.d56, na.action = na.omit, id = Tier.Nr., waves = Trial,
    corstr = "exchangeable")

 Coefficients:
               Estimate Std.err Wald Pr(>|W|)
(Intercept)     -0.3798  0.2896 1.72   0.1897
Day.f6           0.9400  0.3254 8.34   0.0039 **
Trial.rep       -0.0765  0.0934 0.67   0.4126
GruppeHyperoxia -0.1776  0.1953 0.83   0.3631
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Estimated Scale Parameters:
            Estimate Std.err
(Intercept)     1.23   0.165

Correlation: Structure = exchangeable  Link = identity

Estimated Correlation Parameters:
      Estimate Std.err
alpha   -0.062  0.0257
Number of clusters:   12   Maximum cluster size: 8



Analysis of 'Wald statistic' Table
Model: poisson, link: log
Response: re.bait
Terms added sequentially (first to last)

          Df   X2 P(>|Chi|)
Day.f      1 8.47    0.0036 **
Trial.rep  1 0.68    0.4088
Gruppe     1 0.83    0.3631
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

B.2.5 mHB: latency to complete (Day 1–5) [s]




```
   Model df AIC BIC logLik   Test L.Ratio p-value
m      1 21 197 268  -77.5
m0     2 22 142 216  -48.8 1 vs 2    57.3  <.0001
m1     3 25 147 232  -48.4 2 vs 3     0.9   0.828
m2     4 26 146 234  -47.2 3 vs 4     2.4   0.118
m3     5 41 161 300  -39.4 4 vs 5    15.5   0.415


Generalized least squares fit by REML
  Model: log10(Globalzeit) ~ Day.f * Trial.rep * Gruppe
  Data: mHB.LO.d15
  AIC BIC logLik
  142 216  -48.8

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
 Rho
0.33

Coefficients:
                                      Value Std.Error t-value p-value
(Intercept)                           2.332    0.1421   16.42  0.0000
Day.f2                               -0.559    0.1743   -3.21  0.0015
Day.f3                               -0.695    0.1743   -3.99  0.0001
Day.f4                               -0.787    0.1758   -4.48  0.0000
Day.f5                               -1.100    0.1743   -6.31  0.0000
Trial.rep                            -0.168    0.0450   -3.74  0.0002
GruppeHyperoxia                       0.046    0.2009    0.23  0.8182
Day.f2:Trial.rep                      0.082    0.0637    1.29  0.1990
Day.f3:Trial.rep                      0.115    0.0637    1.80  0.0725
Day.f4:Trial.rep                      0.062    0.0651    0.95  0.3436
Day.f5:Trial.rep                      0.145    0.0637    2.28  0.0238
Day.f2:GruppeHyperoxia                0.215    0.2465    0.87  0.3850
Day.f3:GruppeHyperoxia               -0.007    0.2465   -0.03  0.9782
Day.f4:GruppeHyperoxia                0.127    0.2476    0.51  0.6094
Day.f5:GruppeHyperoxia                0.176    0.2465    0.71  0.4767
Trial.rep:GruppeHyperoxia             0.004    0.0637    0.06  0.9484
Day.f2:Trial.rep:GruppeHyperoxia     -0.074    0.0900   -0.82  0.4122
Day.f3:Trial.rep:GruppeHyperoxia     -0.017    0.0900   -0.19  0.8501
Day.f4:Trial.rep:GruppeHyperoxia      0.016    0.0910    0.17  0.8625
Day.f5:Trial.rep:GruppeHyperoxia     -0.052    0.0900   -0.58  0.5641

Standardized residuals:
     Min       Q1      Med       Q3      Max
-2.23037 -0.66127  0.00188  0.62089  2.86020

Residual standard error: 0.301
Degrees of freedom: 239 total; 219 residual

Denom. DF: 219
                    numDF F-value p-value
(Intercept)             1   269.5  <.0001
Day.f                   4    10.7  <.0001
Trial.rep               1    14.0  0.0002
Gruppe                  1     0.1  0.8182
Day.f:Trial.rep         4     1.5  0.2040
Day.f:Gruppe            4     0.3  0.8530
Trial.rep:Gruppe        1     0.0  0.9484
Day.f:Trial.rep:Gruppe  4     0.3  0.8553


Generalized least squares fit by REML
  Model: log10(Globalzeit) ~ Day.f + Trial.rep + Gruppe
  Data: mHB.LO.d15
   AIC BIC logLik
  82.5 114  -32.3

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.326

Coefficients:
                Value Std.Error t-value p-value
(Intercept)     2.137    0.0882   24.22   0.000
Day.f2         -0.339    0.0507   -6.70   0.000
Day.f3         -0.432    0.0507   -8.53   0.000
Day.f4         -0.548    0.0509  -10.76   0.000
Day.f5         -0.715    0.0507  -14.12   0.000
Trial.rep      -0.098    0.0144   -6.79   0.000
GruppeHyperoxia 0.094    0.1046    0.90   0.368

Standardized residuals:
    Min      Q1     Med      Q3     Max
-2.4713 -0.6734  0.0702  0.6663  3.0379

Residual standard error: 0.302
Degrees of freedom: 239 total; 232 residual


Denom. DF: 232
          numDF F-value p-value
(Intercept)     1     587  <.0001
Day.f           4      56  <.0001
Trial.rep       1      46  <.0001
Gruppe          1       1   0.368
```

B.2.6 mHB: latency to complete (Day 5–6) [s]




```
   Model df  AIC   BIC logLik   Test L.Ratio p-value
m      1  9 67.8  90.1  -24.9
m0     2 10 52.5  77.3  -16.2 1 vs 2   17.33  <.0001
m1     3 13 57.8  90.0  -15.9 2 vs 3    0.66   0.883
m2     4 11 54.3  81.6  -16.1 3 vs 4    0.49   0.783
m3     5 17 65.4 107.6  -15.7 4 vs 5    0.87   0.990
```

```
Generalized least squares fit by REML
  Model: log10(Globalzeit) ~ Day.f * Trial.rep * Gruppe
  Data: mHB.LO.d56
   AIC  BIC logLik
  52.5 77.3  -16.2

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.338

Coefficients:
                                Value Std.Error t-value p-value
(Intercept)                     1.232    0.1323    9.31  0.0000
Day.f6                          0.330    0.1615    2.04  0.0444
Trial.rep                      -0.023    0.0417   -0.56  0.5783
GruppeHyperoxia                 0.222    0.1871    1.19  0.2386
Day.f6:Trial.rep               -0.013    0.0590   -0.21  0.8325
Day.f6:GruppeHyperoxia         -0.133    0.2285   -0.58  0.5615
Trial.rep:GruppeHyperoxia      -0.048    0.0590   -0.81  0.4192
Day.f6:Trial.rep:GruppeHyperoxia  0.013    0.0834    0.15  0.8784

Standardized residuals:
   Min     Q1    Med     Q3    Max
-2.391 -0.756  0.107  0.669  2.515

Residual standard error: 0.281
Degrees of freedom: 96 total; 88 residual


Denom. DF: 88
                  numDF F-value p-value
(Intercept)           1    86.7  <.0001
Day.f                 1     4.2  0.0444
```

```
Trial.rep                 1     0.3  0.5783
Gruppe                    1     1.4  0.2386
Day.f:Trial.rep           1     0.0  0.8325
Day.f:Gruppe              1     0.3  0.5615
Trial.rep:Gruppe          1     0.7  0.4192
Day.f:Trial.rep:Gruppe    1     0.0  0.8784
```

```
Generalized least squares fit by REML
  Model: log10(Globalzeit) ~ Day.f + Trial.rep + Gruppe
  Data: mHB.LO.d56
   AIC  BIC logLik
  31.6 46.7   -9.8

Correlation Structure: Compound symmetry
 Formula: ~1 | Tier.Nr.
 Parameter estimate(s):
  Rho
0.345

Coefficients:
               Value Std.Error t-value p-value
(Intercept)    1.325    0.0934   14.18  0.0000
Day.f6         0.248    0.0461    5.37  0.0000
Trial.rep     -0.050    0.0206   -2.44  0.0167
GruppeHyperoxia  0.052    0.1052    0.49  0.6243

Standardized residuals:
    Min      Q1     Med      Q3     Max
-2.2038 -0.7491  0.0668  0.5881  2.5960

Residual standard error: 0.279
Degrees of freedom: 96 total; 92 residual


Denom. DF: 92
            numDF F-value p-value
(Intercept)     1   201.0  <.0001
Day.f           1    28.8  <.0001
Trial.rep       1     5.9  0.0167
Gruppe          1     0.2  0.6243
```

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