Cell-mediated immune ontogeny is affected by sex but not environmental context in a long-lived primate species

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Author contribution statement

VB, CD, GH, and BF: conception and design. JMGS, MK, SL, GH, and BF: sample acquisition. VB and MH: sample analysis. VB: statistical analysis. All authors were involved in interpretation of the data. VB, CD, GH, BF and MH: drafting of the manuscript. All authors revised, reviewed, and approved the final version of the manuscript.

Keywords

Ecoimmunology, macroimmunology, immune ontogeny, Bonobo, Pan paniscus, Development of immunocompetence, Neopterin

Abstract

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Ecoimmunology conceptualizes the role of immunity in shaping life history in a natural context. Within ecoimmunology, macroimmunology is a framework that explains the effects of habitat and spatial differences on variation in immune phenotypes across populations. But immune ontogeny – the development of the immune system across an individual life span – has received little attention within these two frameworks. Here, we investigated how immune ontogeny from birth until adulthood is affected by age, sex, and developmental environment in a long-lived primate species, the bonobo. We found a progressive, significant decline of urinary neopterin levels, a marker for the cell-mediated immune response, from birth until five years in both sexes. However, the overall pattern of age-related neopterin changes is sex-specific, with males having higher urinary neopterin levels than females in the first three years of life, and females having higher levels than males between six to eight years. Environmental condition (zoo-housed vs. wild) did not influence neopterin levels, nor did age-related changes in neopterin levels differ between environments. Therefore, the post-natal development of cell-mediated immune ontogeny in this long-lived primate species is sex-specific but does not show plasticity in response to environmental conditions. Our results indicate that cell-mediated immune ontogeny follows a stereotypic and probably a genetically determined pattern that is not affected by environmental differences in pathogen exposure and energy availability. However, sex is an important, yet often overlooked factor shaping patterns of immune ontogeny. Investigating the causes and consequences of variation in immunity throughout life is critical for our understanding of life-history evolution and strategies, mechanisms of sexual selection, and population dynamics with respect to pathogen susceptibility. General descriptions of sex-specific immune ontogeny are a crucial step in this direction, but they need to be investigated in the context of a species' ecology and evolutionary history.

Contribution to the field

Our results suggest that immune functioning shifts from cell-mediated to humoral responses in the first years of life in a stereotypical pattern that is unaffected by environmental context but differs between the sexes. This would propose that changes in cell-mediated immunity during immune ontogeny follow probably a genetically determined pattern which is unaffected by environmental factors. Our results propose that sex is an important, hitherto overlooked factor shaping patterns of immune ontogeny. We argue that sex biases in maternal investment and changes in androgenic and estrogenic hormone levels associated with the onset of sexual maturation are drivers for these differences in cell-mediated immune ontogeny. Macroimmunological differences between species can be caused by genetic differences in immunity, environmental pathogen exposure, and interactions between these factors. This finding suggests that a species' ecology and evolutionary history should be considered when interpreting species differences in immune functioning. Our results broaden the understanding of variation in immunity, which is critical for our understanding of evolutionary adaptations, life-history, and population dynamics.

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Ethics statements

Studies involving animal subjects

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Abstract

Ecoimmunology conceptualizes the role of immunity in shaping life history in a natural context. Within ecoimmunology, macroimmunology is a framework that explains the effects of habitat and spatial differences on variation in immune phenotypes across populations. But immune ontogeny – the development of the immune system across an individual life span – has received little attention within these two frameworks. Here, we investigated how immune ontogeny from birth until adulthood is affected by age, sex, and developmental environment in a long-lived primate species, the bonobo. We found a progressive, significant decline of urinary neopterin levels, a marker for the cell-mediated immune response, from birth until five years in both sexes. However, the overall pattern of age-related neopterin changes is sex-specific, with males having higher urinary neopterin levels than females in the first three years of life, and females having higher levels than males between six to eight years. Environmental condition (zoo-housed vs. wild) did not influence neopterin levels, nor did age-related changes in neopterin levels differ between environments. Therefore, the post-natal development of cell-mediated immune ontogeny in this long-lived primate species is sex-specific but does not show plasticity in response to environmental conditions. Our results indicate that cell-mediated immune ontogeny follows a stereotypic and probably a genetically determined pattern that is not affected by environmental differences in pathogen exposure and energy availability. However, sex is an important, yet often overlooked factor shaping patterns of immune ontogeny. Investigating the causes and consequences of variation in immunity throughout life is critical for our understanding of life-history evolution and strategies, mechanisms of sexual selection, and population dynamics with respect to pathogen susceptibility. General descriptions of sex-specific immune ontogeny are a crucial step in this direction, but they need to be investigated in the context of a species' ecology and evolutionary history.

1 Introduction

Ecoimmunology uses an integrative approach to estimate costs, benefits, and fitness consequences of different immune defense strategies, and relates variation in immune responses to phylogeny, sociality, and ecology (Martin et al., 2006; Demas and Nelson, 2012; Schoenle et al., 2018). Since the emergence of this discipline, research has focused on how immunity – the capacity to resist a particular pathogen – affects life history strategies within species (Brock et al., 2014). Macroimmunology is a framework within ecoimmunology that considers habitat- and spatial-specific differences in immune phenotypes across populations (Becker et al., 2020; Forbes, 2020). While previous studies have focused on variation in immunity of adult individuals, immune ontogeny – the development of the immune system across an individuals' life span – as a determining factor for variation and plasticity remained largely unexplored (Goenka and Kollmann, 2015). However, preliminary evidence suggests that immunity, but also exposure to immune challenges, can vary across life stages (Beirne et al., 2016; Peters et al., 2019). Given that many developmental changes vary with environment and sex (Martin et al., 2006; Love et al., 2008), it is plausible that these parameters also affect immune ontogeny.

On an ultimate level, variation in immune ontogeny is assumed to be affected by a species' pace-of-life. Long-lived species are expected to favor investment into long-term immune strategies that have high initial metabolic costs (Martin et al., 2006). So far, studies on the influence of environment and sex on ontogenetic changes in immunity have focused on species with short lifespans and fast pace-of-life. In long-lived species, long developmental periods provide more time for developmentally mediated, adaptive adjustments in life-history trade-offs in response to environmental factors than in short-lived species. Those adjustments can then lead to a larger variation of functional immune phenotypes (Simon et al., 2015; McDade et al., 2016). In this regard, humans are the best studied long-lived species so far, but many aspects of human immunology have only been studied in the context of pathologies (McDade, 2003; Martin et al., 2006; Tieleman, 2018), and studies documenting the variation in "healthy" immune ontogeny are scarce and often focus only on the first year of life. Like in humans, the life-histories of Great apes (*Hominoidea*), humans closest living relatives, are characterized by slow ontogenetic development, an extended period of immaturity, and a long and differentiated phase of adulthood (Robson and Wood, 2008). Therefore, Great apes are an ideal model taxon to investigate sex-specificities and environmental impacts on immune ontogeny that might explain variation in macroimmunology within and between populations. This will facilitate our understanding of immune ontogeny and its effects on adult immunity in our own species.

In vertebrates, the immune system consists of an innate immunity (non-specific immune response) and an adaptive / acquired immunity (specific immune response). Generally, innate immunity is mobilized quickly and provides resistance to a wide range of pathogens without specific antigen recognition. In contrast, adaptive immunity recognizes and targets specific antigens and develops immunological memory (McDade et al., 2016). Moreover, these immune responses differ in function, evolutionary history, and metabolic costs. Innate immunity is metabolically "cheaper" and phylogenetically older than adaptive immunity (McKean and Lazzaro, 2011). Both innate and adaptive immunity have two response trajectories each, a cell-mediated and a humoral response (Murphy and Weaver, 2018). Here, we will focus on cell-mediated responses. Cell-mediated responses include, for example, the activation of macrophages. They are activated by the release of interferon-gamma by type 1 helper T cells (Th1). Cell-mediated immunity targets *intra*cellular pathogens such as all viruses (e.g., *Human alphaherpesvirus 3*), certain bacteria (e.g., *Mycobacterium leprae* and *M. tuberculosis*), and certain protozoa (e.g., *Plasmodium falciparum* and *Leishmania spp.*) (Thakur et al., 2019).

Methods to measure immune responses in vertebrates have mainly used blood samples as their analytical matrix. Blood samples can be difficult to obtain in field studies and / or from animals that cannot be caught to obtain blood samples. Recently, techniques to monitor immune responses have been validated for use in non-invasively collected samples such as feces and urine (Higham et al., 2015, 2020; Behringer et al., 2017; Gesquiere et al., 2020). Specifically, the activation of cell-mediated immunity can be assessed through the measurement of neopterin, a biomarker produced by monocyte-derived macrophages and dendritic cells upon stimulation with Th1-derived interferon gamma. Neopterin can be readily measured in blood and urine (Fuchs et al., 1992; Hoffmann et al., 2003; Winkler et al., 2003a). In humans, children have higher urinary neopterin levels than adults (Fuchs et al., 1992; Winkler et al., 2003a; Girgin et al., 2012), and neopterin values of children between 0-5 years of age are twice as high as levels of children between 6-10 years of age (Girgin et al., 2012). By comparing urinary neopterin level changes and its age-related patterns between sexes and different environmental conditions we are thus able to study aspects of immune ontogeny.

Immune system responses of immature and adult individuals differ in quantity and functionality, but age-related differences of immunity level out with increasing age (Dowling and Levy, 2014; Simon et al., 2015; Georgountzou and Papadopoulos, 2017). After birth, infants depend mainly on innate immunity, because their exposure to antigens in utero is low or absent (PrabhuDas et al., 2011; Dowling and Levy, 2014). During the postnatal phase, when individuals are exposed to environmental pathogens, they require distinct immune responses and the immune system becomes less tolerant in response to environmental pathogen exposure (West, 2002; Goenka and Kollmann, 2015; Simon et al., 2015). In addition, the developing immune system of newborn mammals is facilitated by achieving passive protection through maternal antibodies transferred from mother to infant during lactation (Hasselquist and Nilsson, 2009). As individuals mature further, the immune system provides broader and more specific protection. In humans, young adults suffer fewer infections than immature individuals (Simon et al., 2015; Georgountzou and Papadopoulos, 2017), and a very recent study corroborated this by showing a peak in immune functioning between 5-14 years of age (Glynn and Moss, 2020). Thus, there is clear evidence for ontogenetic changes in immune functioning in humans and some medical research model species, although detailed information on age-related pattern in immunity is lacking for most species, especially long-lived ones.

Macroimmunology considers variation of environmental parameters such as resource availability, pathogen exposure, and pathogen diversity on a spatial scale and relates this information to variation in immune system functioning (Becker et al., 2020; Forbes, 2020). During ontogeny, the costs and benefits associated with these certain aspects of immune functioning may vary with environmental parameters, which can lead to different immune phenotypes (Brock et al., 2014; Tieleman, 2018; Becker et al., 2020). For example, malnutrition is known to impair immune functioning (e.g., (Chandra, 1997; Marcos et al., 2003; McDade, 2003)), and therefore, individuals in nutritionally limited environments are expected to favor investment into energetically cheaper innate immunity rather than into costly adaptive immunity (McDade et al., 2016). Furthermore, the intensity and diversity of pathogen exposure during ontogeny is hypothesized to be an important driver determining adult immunity (McDade, 2003, 2012). Exposure to fewer but probably novel pathogens is expected to favor innate over adaptive immune responses. Correspondingly, populations with high, but more familiar pathogens, are predicted to develop specific immunity earlier (McDade et al., 2016). Markers of innate immunity were indeed found to be higher in humans living in habitats with high rather than low pathogen exposure (McDade, 2003; Teran et al., 2011). Similar results were found in adult chimpanzees where animals living in the wild showed generally higher neopterin levels than animals living in a zoo environment, which likely have less immune challenges (Behringer et al., 2019). Comparative studies like the latter are therefore ideal to investigate factors that are likely to shape

differences in immune ontogeny. Compared to wild populations, the diet of zoo-housed primates is higher in quality and quantity and we can expect that this difference in energy supply affects energetic investment into immune system functioning during ontogeny and leads to differences in adult immune functioning. In addition, zoo-housed populations usually receive intense medical care when infected, and this may also affect the strength and quality of their immunity (Courtenay and Santow, 1989). Although wild populations are probably exposed to a higher quantity of pathogens, zoo-housed animals are in close contact to humans which may increase the risk of transmission of zoonotic infections and exposure to new pathogens. This is particularly relevant for Great apes because of their high susceptibility to human pathogens (Calvignac-Spencer et al., 2012; Dunay et al., 2018). This difference in quality and quantity of pathogen exposure is also expected to affect immune ontogeny. A comparison of the immune ontogeny of wild and zoo-housed populations is therefore ideal to test the predictions of the macroimmunological framework.

In humans and other mammals, immunity tends to be sex-biased with adult females having fewer infections and stronger antibody responses, but also greater vulnerability to autoimmune diseases than males (e.g., (Klein and Roberts, 2010; Klein and Flanagan, 2016; Metcalf et al., 2020) but see (Kelly et al., 2018)). Cell-mediated immune responses also differ between males and females. For example, females have higher Th2 responses than males (Klein and Flanagan, 2016). These differences in immunity are seen as sex-specific trade-offs in resource allocation favoring immunity or reproduction (Zuk, 2009). Sex hormones with higher concentrations in females (i.e., estrogens) enhance immune functioning, but those with higher concentrations in males (i.e., androgens) are thought to negatively affect immune responses (Zuk, 2009; Fischer et al., 2015; Kelly et al., 2018) and support behaviors that make adult males more susceptible to infections (Klein, 2000). While sex differences in adult immunity are known, information about sex-specific immune ontogeny is rare (Fish, 2008; Klein and Flanagan, 2016). For example, in pre-pubertal humans, inflammatory processes are higher in boys than in girls, but after puberty this relationship is reversed (Yang and Kozloski, 2011; Klein and Flanagan, 2016). So far, the few studies available indicate that immune ontogeny in humans is sex-specific and we can therefore expect to find similar differences in the immune ontogeny in non-human primate species.

Bonobos (*Pan paniscus*) and chimpanzees (*P. troglodytes*) are sister species and are humans' closest living relatives. They are often used as living models for reconstructing the developmental characteristics of our last common ancestor (Bastir and Rosas, 2004). Like humans, bonobos mature slowly, delay reproduction, and have a long life expectancy (Robson and Wood, 2008; Barrickman, 2016). Maximum life expectancy in the wild is estimated to be 45 years, while the oldest zoo-housed bonobo is estimated to be 69 in 2020 (Lowenstine et al., 2015; Stevens, 2020). In apes, maturation until adulthood accounts for one third of the total lifespan, the period between the onset of sexual maturation and first reproduction is relatively long, and there is a considerable gap between the time when adult body size is reached and the emergence of reproductive competence (Lancaster and Hamburg, 2008; Walker et al., 2018). Given the importance of developmental changes for the reconstruction of phylogenetic trends, there is increasing interest in the postnatal development of bonobos and chimpanzees (Behringer et al., in press; Hare and Yamamoto, 2015; Lee et al., 2020). Yet, information on immune ontogeny in these species is rare. Bonobos are particularly interesting because recent studies revealed that their major histocompatibility complex (MHC) class I profile has undergone severe selection pressures that have reduced its variability (Prado-Martinez et al., 2013; de Groot et al., 2017, 2018; Maibach et al., 2017; Wroblewski et al., 2017; Maibach and Vigilant, 2019). Moreover, the MHC-B repertoire of bonobos was found to be diminished even more than in humans and chimpanzees (de Groot et al., 2017; Maibach et al., 2017; Wroblewski et al., 2017). It is assumed that

such a reduction in genetic diversity of the MHC influence the functioning of immunity (Sommer, 2005; de Groot et al., 2017).

In order to understand immune ontogeny in relation to ecological pressures and across evolutionary times, it is necessary to investigate free-living, genetically diverse, and energetically limited populations of animals using an ecoimmunological and macroimmunological approach (Demas and Nelson, 2012; Forbes, 2020). Here we investigated the effects of age, environment (wild vs. zoo-housed), and sex on cell-mediated immune ontogeny in bonobos, a long-lived primate species that is closely related to humans. We measured urinary neopterin in individuals ranging from birth until 18 years of age. Using information from human studies as a conceptual benchmark, we tested the following predictions: 1. Age: If the immune system shifts from cell-mediated to humoral during the first years of life, neopterin levels will be elevated in infant bonobos and decline afterwards. 2. Environment: 2a) If pathogen exposure is higher in natural habitats, we expect neopterin levels to be higher in wild than in zoo-housed bonobos. 2b) Moreover, if food resources are a driving force influencing the pace of immune ontogeny, zoo-housed individuals are expected to show an earlier decline of neopterin levels than wild ones, indicating a faster immune ontogeny. 3. Sex: 3a) If the immune system of males relies more on cell-mediated than humoral responses, we expect higher neopterin levels in males than in females. 3b) And if sex-specific developmental processes, such as the increase of sex steroid hormones with the beginning of sexual maturation, affect immune ontogeny, we expect neopterin levels to decline earlier in females than in males, independent of the environment.

2 Material and Methods

Study sites and subjects

Between June 2008 and October 2019, 597 urine samples (female: 375; male: 222) were collected from two wild bonobo communities (Bompusa East and Bompusa West communities) at the LuiKotale field site, Democratic Republic of the Congo (Hohmann and Fruth, 2003). All subjects were habituated to human presence before the start of the study period, and all were individually known. Samples represent 34 individual females (average: 11 samples per individual) and 21 individual males (average: 11 samples per individual). For wild subjects, the month and year of birth was known for 13 individuals, and for these we set the date of birth to the 15th of the respective month. For 12 individuals only the year of birth was known, and we set the birth date to June 15th of the respective year. For the remaining 30 individuals' exact birthdates are known. Zoo-housed bonobos were sampled between January 2012 and September 2018, and 237 urine samples (female: 137; male: 100) were collected in nine different zoos. These samples include 49 individual females (average: three samples per individual) and 26 individual males (average: four samples per individual). All zoo-housed bonobos were of known age, and were housed in mixed-sex groups of different sizes. Age range was 0 to 18 years of age for both populations (zoo average: eight years; wild average: five years). All samples were collected at a time when individuals did not show symptoms of infection or disease (e.g., running noses or eyes, coughing or sneezing, visible wounds).

Urine collection

Urine samples were collected opportunistically throughout the day between 6:00h and 20:00h. Samples were collected on plastic sheets or the ground floor for zoo-housed bonobos, and from vegetation for wild animals. Samples were protected from direct sunlight, to avoid neopterin degradation (Fuchs et al., 1992; Behringer et al., 2017), and were excluded when contamination with feces was detected. In the field, urine samples were frozen in liquid nitrogen upon arrival at camp. Zoo samples were frozen

immediately after collection. All urine samples were transported frozen to the Max Planck Institute for Evolutionary Anthropology in Leipzig, Germany, and later to the German Primate Center, Goettingen, Germany for analysis.

Sample preparation and neopterin measurement

Prior to analysis, urine samples were thawed, vortex-mixed for ten seconds, and subsequently centrifuged for five minutes. We measured neopterin levels with a commercial neopterin ELISA kit (Neopterin ELISA, Ref. RE59321, IBL International GmbH, Hamburg, Germany), previously validated for use with bonobo urine (Behringer et al., 2017). Initially, we determined specific gravity (SG) in all samples using a digital handheld refractometer (TEC, Ober-Ramstadt, Germany). SG population average was 1.005 for zoo-housed individuals, and 1.011 in wild bonobos. Highly dilute samples with a SG of <1.003 were excluded from the data set (N = 28). Prior to neopterin measurement, urine samples were diluted (1:10 to 1:200 depending on SG) with the provided assay buffer, and samples were measured in duplicate according to the supplier's instructions (for details see Behringer et al. 2017). Inter-assay variation for high- and low-value quality controls was 5.8 % and 6.1 % (N = 28 assays), respectively. Intra-assay variation was 6.4 % (N = 42 urine samples). All neopterin concentrations were adjusted for variation in urine volume and concentration using the samples´ specific gravity (SG) value (Miller et al., 2004). Final neopterin concentrations are expressed in ng/ml corrected for SG.

Ethics

Sample collection was non-invasive. The protocol for urine sample collection in zoo-housed individuals was approved by the authorities of each zoo and supported by the coordinators of the bonobo studbook. Permission to conduct the research at the LuiKotale field site was granted by the Institut Congolais pour la Conservation de la Nature (ICCN).

Statistics

Expecting age-related but non-linear patterns in urinary neopterin levels, we fitted a generalized additive model (GAM) (Wood, 2011) with gaussian-identity link function using R (R Development Core Team, 2008) to our data. The GAM is composed of a sum of smooth functions of covariates and provides a structure for generalizing a general linear model by allowing additivity of non-linear functions of the variables (Wood, 2004, 2017; Ravindra et al., 2019). We used the R package "mgcv" version 1.8–27 (Wood, 2017) to investigate age-related changes in urinary neopterin from birth through early adulthood (18 years of age). Neopterin levels were log-transformed. Sex and environmental condition (zoo-housed, wild) were included as ordered factors, and time of sample collection as a control predictor. To model changes in neopterin levels with age, we included age as a smooth term with a penalized cubic regression. We included interaction-like terms of age with sex, and age with environmental condition by using the "by" argument to the smooth term (Wood, 2017). Because sex and environmental condition are ordered factors, an indicator vector is generated for each level, but not for the first level of the ordered factor. Individual identity and zoo / community were included as a random effect to account for unequal and repeated sampling of individuals across ages and environments. The basis dimension, k was set to 10. The choice of basis dimensions determines the maximum possible degrees of freedom allowed for each model term (Wood, 2017). To control for the potential influence of time of sample collection, this parameter was included as a fixed effect in the model as a control predictor. Diagnostics were done using "gam.check". Model assumptions were assessed by visual inspections of a histogram, a q–q plot of the residuals, and by plotting residuals

against fitted values and basis dimension. The lowest k-index<1 was 0.43 and all other model assumptions were met. Concurvity, the situation where a smooth term can be approximated by some combination of the others, was not an issue.

3 Results

From birth onwards, urinary neopterin levels declined significantly and progressively with age until the age of five and then remained largely stable at older ages (Figures 1 and 2). Environmental conditions did not explain differences in neopterin levels in non-adult bonobos (Table 1, Figure 1, $P_{environment} = 0.925$) or changes in urinary neopterin levels with age (Table 1 smooth terms, Figure 1). Overall, urinary neopterin levels were more variable in females than in males. Sex did not predict neopterin levels in our dataset (Table 1, $P_{sex} = 0.805$), but the smooth function for the interaction-like term for age with sex was significant. This indicates that age-related changes of urinary neopterin levels differ between the sexes (Table 1 smooth terms, Figure 2). Independent of the environmental context, urinary neopterin levels were significantly higher in males than in females during the first three years of life. This pattern reversed at around six years when neopterin levels of females increased successively until they became significantly higher than levels of males (Figure 3).

4 Discussion

In this study we used urinary neopterin as a biomarker to investigate the effects of age, environment, and sex on cell-mediated immune ontogeny across the first 18 years of life in the bonobo, a long-lived primate species with a slow life-history. As predicted, urinary neopterin values progressively declined from birth until the age of approximately five years, after which they remained largely stable at low levels. In contrast to our expectations, urinary neopterin levels were not influenced by environmental condition nor were changes with age environment-specific. However, sex influenced the pattern of age-related neopterin levels, with males having higher neopterin levels than females in the first three years of life and lower levels between 6-8 years.

Neopterin levels decline with age

In wild and zoo-housed bonobos, urinary neopterin levels declined after birth and stabilized between four to five years of age. These age-related changes in urinary neopterin levels of immature bonobos are in line with data from humans (Fuchs et al., 1992; Winkler et al., 2003a; Girgin et al., 2012). The decline in neopterin levels may represent the shift from predominantly cell-mediated towards more humoral immune responses. In humans, in the first years after birth, antibody responses to pathogens are delayed, diminished, and less persistent than those in adults (Goenka and Kollmann, 2015; Georgountzou and Papadopoulos, 2017). During this time, infants still rely mostly on their innate immunity (McDade et al., 2016), and are also protected by maternal antibodies that are transferred through breast milk during lactation (Hasselquist and Nilsson, 2009; Goenka and Kollmann, 2015). Although T- and B-cells are already abundant at birth, their phenotypes are predominantly immature (McDade, 2003; Goenka and Kollmann, 2015). As these cells mature and differentiate with age, the developing immune system balances innate and adaptive responses (Teran et al., 2011). Therefore, we conclude that the decline in urinary neopterin levels during the first give years of life represents the shift from cell-mediated to humoral immune responses and can be used as a biomarker to monitor immune ontogeny in wild and captive populations.

Neopterin is produced by activated macrophages and monocytes. These immune cells are stimulated by the cytokine interferon gamma (Fuchs et al., 1993; Murr et al., 2002), and probably not by other factors (Mosser and Edwards, 2008). Importantly, interferon gamma is produced by adaptive immune

cells (specifically T helper cells type 1) in response to intracellular infections (Murr et al., 2002) and by innate immune cells such as natural killer cells (Yu et al., 2006). Therefore, neopterin is an essential component of innate and adaptive immune responses. Both types of immune responses will be challenged frequently during early development when the organism is confronted with pathogens for the first time (Winkler et al., 2003a, 2003b). Consequently, changes in neopterin levels during ontogeny reflect the activation of cell-mediated immunity but cannot be used to distinguish between the activation of innate and adaptive immune responses. The higher urinary neopterin levels in the first five years of life in bonobos therefore indicate that infant bonobos rely predominantly on cell-mediated immunity during infancy and that humoral immune responses become more important with time. A more differentiated view of immune ontogeny requires additional markers of specific immune response. However, many of these markers can so far only be measured in blood samples. Our results on bonobo immune ontogeny are not only interesting in the context of ecoimmunology, but they also have practical implications for conservation efforts of this endangered species given that the transmission of diseases, particularly respiratory diseases, threatens wild and zoo-housed ape populations (King et al., 2005; Leendertz et al., 2006; Sakamaki et al., 2009; Jones et al., 2011; Strong et al., 2016; Grützmacher et al., 2018; Ryu et al., 2020). As immature apes are important vectors of disease transmission within social groups (Kuehl et al., 2008), identification of sensitive windows during immune ontogeny can support management and conservation efforts.

Neopterin levels are independent of environmental context

Contrary to our predictions, cell-mediated immune ontogeny in bonobos was not affected by environmental conditions. Urinary neopterin levels in wild and zoo-housed bonobos showed comparable changes with age, suggesting that these aspects of immune ontogeny during early life are stereotypic and more determined by genetic factors than modulated by environmental factors. This result corresponds with findings in humans. Only small differences in immune functioning (blood cultures and cytokine assays) were found between children growing up in urban versus rural environments in the Tropics (Teran et al., 2011), which are also expected to differ in pathogen exposure. In the first three months of life, early immune ontogeny was found to follow a stereotypical pattern, and within this time, pre-term and term children converged on a shared trajectory (Olin et al., 2018). Our data suggest that in bonobos cell-mediated immune ontogeny follows a stereotypic pattern for a much longer time, suggesting that studies investigating human immune ontogeny should include longer times periods.

The finding that urinary neopterin levels in wild and zoo-housed bonobos are similar was unexpected because neopterin levels of adult wild chimpanzees were found to be significantly higher than those of zoo-housed individuals, and this finding was assumed to reflect differences in pathogen exposure (Behringer et al., 2019). However, urinary neopterin levels of immature bonobos (both zoo-housed and wild around 360 ng/mL corr. SG) in our study were on average nearly half of those of immature wild chimpanzees (average 759 (ng/mL corr. SG)) (Löhrich et al., 2018), suggesting inter-specific differences in aspects of bonobo and chimpanzee ecoimmunology. Generally lower neopterin levels in bonobos could be a species-specific adaption that is the result of selective pressures unique to bonobos. Higher neopterin levels in wild chimpanzees compared to bonobos might reflect differences in virus prevalence and immune resistance. For example, African apes differ in the susceptibility to *Plasmodium* parasite species which cause malaria. In humans, malaria infections stimulate an increase in neopterin levels as part of the cell-mediated immune response against these intracellular parasites (Brown et al., 1990, 1991; Fuchs et al., 1992; Biemba et al., 2000; te Witt et al., 2010). Wild chimpanzees also suffer from malaria infections, caused by a diversity of *Plasmodium* species (De Nys

et al., 2014; Otto et al., 2014; Herbert et al., 2015), and likely have the same neopterin response to this infection as humans.

Although, bonobos live in an area of high malaria prevalence, malaria infections are almost absent in bonobos (Krief et al., 2010; Liu et al., 2017), presumably due to the presence of particular malaria protecting MHC allotypes (Sanchez-Mazas et al., 2017; de Groot et al., 2018). The low neopterin levels of bonobos reported in this study might therefore reflect this difference in malaria susceptibility. Other viruses that are known to increase neopterin values are also known to differ in prevalence between humans, chimpanzees and bonobos. Neopterin levels are markedly increased in humans infected with human immunodeficiency virus (Fuchs et al., 1992), and a similar immune response is reported for macaques infected with the related simian immunodeficiency virus (SIV) (Heistermann and Higham, 2015). SIV infections are also common and widespread in wild chimpanzees (Gao et al., 1999; Li et al., 2012), but no bonobo sample from the wild has tested positive for SIV (Li et al., 2012). Therefore, low neopterin levels in wild bonobos could also reflect differences in SIV exposure between chimpanzees and bonobos. However, the low urinary neopterin levels reported in our study could also reflect specific conditions of our study population (prevalence, frequency, and type of pathogens), and / or a species-specific trait that has emerged as an adaption to environmental conditions of the central Congo basin. It may reflect a spatial limitation for migration that prevents bonobos from invading novel habitats. Additional studies are therefore needed to determine if this species difference in neopterin levels persists in older adult bonobos, and if acute neopterin responses to specific pathogen encounters differ between wild bonobos and chimpanzees. Examining broad differences in pathogen susceptibility and prevalence between the two species and how they shape species-specific age-related immune patterns will require integrating neopterin measurements with local disease ecology of wild bonobos and chimpanzees.

Based on our findings, we hypothesize that the low levels of urinary neopterin in immature wild bonobos could be explained by a lower prevalence of intracellular pathogens. Additionally, if the bonobo immune system is adapted to pathogens prevalent in their environment (i.e., malaria), then it is possible that infections with these pathogens do not elicit a strong immune / neopterin response (old friends hypothesis, see (Rook et al., 2003)).

The bonobo-chimpanzee-human comparison can serve as a model to investigate consequences of past selection pressures on present immune systems, highlighting extant species-specific differences in immune ontogeny and functioning. Immune system differences between the species might reflect causes or consequence of past environmental changes and isolation of certain populations. These differences may have had far reaching consequences for the adaptive potential to new environments during the evolution of these species. And today, these immunological differences might also impact current risks of extinction with respect to changing disease landscapes with climate change and globalization.

Changes in neopterin levels during ontogeny are sex-specific

We did not find support for our prediction that neopterin levels in males are higher overall compared to females, which was based on a proposed higher reliance of males on cell-mediated immune responses. In line with our result, urinary and serum neopterin were not statically different between large cohorts of healthy boys and girls (Sack et al., 1998; Plata-Nazar et al., 2007; Girgin et al., 2012). However, we found significant differences between male and female neopterin levels at certain age stages during ontogeny. Compared to females, male bonobos had higher neopterin levels during the first three years of life, but lower levels between the ages 6-8 years.

There are multiple of possible explanations for the sex difference in neopterin levels during the first three years of life. Behavioral sex differences can cause differential pathogen exposure and infection risk, and therefore result in differences in immune functioning (Zuk, 2009). Behavioral sex differences that cause differential pathogen exposure are expected to occur only later in life when males engage in more risk taking behaviors (Klein, 2004) that are likely to induce social and energetic stress which in turn may increase the risk of infections (Zuk, 2009). In bonobos, males do not engage in these behaviors during their years when they are highly dependent on their mothers. Therefore, it is unlikely that behavioral sex differences account for the difference in neopterin levels at this age in bonobos. A more ultimate explanation for the difference in neopterin levels before the age of three is that sex-biased maternal investment in offspring occurs through unbalanced, sex-specific provisioning (Trivers and Willard, 1973; Maynard Smith, 1980; Silk, 1983). Indeed, sex-biased maternal investment in offspring until weaning age is a widespread phenomenon in animals (e.g., ape (Boesch, 1997) monkey (Hinde, 2009), voles (Koskela et al., 2004), ungulates (Hewison and Gaillard, 1999)). For example, milk composition in many mammals is sex-specific (Landete-Castillejos et al., 2005; Hinde, 2009; Robert and Braun, 2012). Of particular relevance to immune ontogeny is the finding that human mothers provide less secretory immunoglobulin to sons than to daughters with their milk (Fujita et al., 2019). It can be argued that these kinds of sex-biased maternal investment are also present in bonobos. If such a sex difference exists, it may affect immunity during the first years of life, and might explain the higher cell-mediated immune responses in males than females during lactation age in our study. In support of this hypothesis is the finding that sex differences in neopterin levels diminishes around weaning age in apes (de Lathouwers and Van Elsacker, 2006; Fahy et al., 2014; Bădescu et al., 2016).

Between six to eight years female bonobos had significantly higher neopterin levels than males. This age range corresponds with the beginning of sexual maturation in female zoo-housed bonobos. Female bonobos start sexual maturation, defined by increasing urinary testosterone levels, around the age of five years, which is about two to three years earlier than males (Behringer et al., 2014). Therefore, the age range where higher neopterin levels are seen in females corresponds to the difference in the onset of sexual maturation between the sexes in bonobos. For humans, it is reported that the onset of sexual maturation affects immune responses (Klein and Flanagan, 2016). Specifically, the increases in sex hormone levels with the onset of sexual maturation seem to be potent immune system modulators (Fischer et al., 2015). For example, estradiol increases Th2 type immune responses and pro-inflammatory processes. In contrast, testosterone and progesterone bias the specific cell-mediated immune response towards Th1 type responses and activate anti-inflammatory processes (Fischer et al., 2015; Klein and Flanagan, 2016). It is therefore possible that the increase in testosterone levels in female bonobos at 5-6 years of age stimulates an increase in Th1 type immune activity and that this leads to elevated neopterin levels during this period. Another potential explanation is that female bonobos at this age start to leave their natal groups to briefly join different neighboring groups until they become an established resident in one of them (Lee et al., 2020; Sakamaki et al., 2015). Visiting other groups may expose these females to more various pathogens, because social interactions between members of different groups have the potential to increase the risk of disease transmission (Ryu et al., 2020). We could speculate, that increased neopterin levels at these ages could therefore also reflect the immunological challenges of the migration process in females. However, as the effect was seen in both zoo-housed and wild female bonobos it may be that the effect is not caused by differences in pathogen exposure between the sexes at this age. Our findings support the idea that the decline of cell-mediated immune system responses during development might differ between the sexes in timing and magnitude, and in relation to hormonal changes with the onset of sexual maturation.

5 Conclusion

We investigated the effects of environment and sex on cell-mediated immune ontogeny from birth to adulthood in a long-lived primate using urinary neopterin. Our results suggest that immune functioning shifts from cell-mediated to humoral responses in the first years of life in a stereotypical pattern that is unaffected by environmental context but differs between the sexes. This would propose that changes in cell-mediated immunity during immune ontogeny follow probably a genetically determined pattern which is unaffected by environmental factors. Our finding contrast with the current hypotheses that differences in pathogen exposure and energy availability during ontogeny affect immune ontogeny and drives differences in adult immune functioning. Our results propose that sex is an important, hitherto overlooked factor shaping patterns of immune ontogeny. We argue that sex biases in maternal investment and changes in androgenic and estrogenic hormone levels associated with the onset of sexual maturation are drivers for these differences in cell-mediated immune ontogeny. Macroimmunological differences between species can be caused by genetic differences in immunity, environmental pathogen exposure, and interactions between these factors. This finding suggests that a species' ecology and evolutionary history should be considered when interpreting species differences in immune functioning.

Our findings are relevant for the fields of ecoimmunology and macroimmunology because current hypotheses emphasize environmental factors during ontogeny in shaping adult immune functioning. Our results indicate that genetic and sex-specific processes are also important and should therefore be considered in future studies. This can be done by integrating hormone measurements, behavioral observations, and specific pathogen exposure with immunological data.

The bonobo-chimpanzee-human comparison can serve as a model to investigate consequences of past selection pressures on immune systems, highlighting extant species-specific differences in immune ontogeny and functioning. These underlying differences may have had far reaching consequences for the adaptive potential during the evolution of these species, and impact current extinction risk with respect to changing disease landscapes with climate change, globalization, and increasing human contact.

We are aware that our study has looked at only one aspect of immunity with a functional biomarker that indicates activity of only a part of the cell-mediated immune response. Therefore, your conclusions are limited to this aspect of immune ontogeny, and do not necessarily translate directly to other aspects of immunity. In the future, studies should try to measure a complementary set of biomarkers that gives specific information about certain aspects of immune functioning. This means that current methods have to be adapted and extended for their use in non-invasive samples.

Investigating the causes and consequences of variation in immunity throughout life is critical for our understanding of life-history, sexual selection and population dynamics. Insights into immune ontogeny are a crucial step in this direction, but they need to be investigated in the context of a species' ecology and evolutionary history. The frameworks of ecoimmunology and macroimmunology offer crucial guidance for these endeavors, and clinical research about the developmental origins of health and disease can benefit by integrating these different viewpoints.

Data availability statement

The datasets generated for this study are available on request to the corresponding author.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

VB, CD, GH, and BF: conception and design. JMGS, MK, SL, GH, and BF: sample acquisition. VB and MH: sample analysis. VB: statistical analysis. All authors were involved in interpretation of the data. VB, CD, GH, BF and MH: drafting of the manuscript. All authors revised, reviewed, and approved the final version of the manuscript.

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Table 1: Estimates of parametric coefficients and effective degrees of freedom of smooth terms for urinary neopterin levels in bonobos between birth and 18 years of age (generalized additive model, $R^2 = 0.21$, deviance explained = 23.8%).

Parametric coefficients	Estimate	SE	P-value
Intercept	5.26	0.05	**<0.001**
Environmental condition (zoo)	-0.01	0.09	0.925
Sex (male)	-0.02	0.07	0.805
Daytime	-0.02	0.03	0.494
Smooth terms	**Edf**		**P-value**
Age	3.71		**<0.001**
Age * environment (zoo)	1.00		0.533
Age * sex (male)	4.09		**0.004**

858 **Figure caption**

859 Figure 1: Log-transformed urinary neopterin levels corrected for specific gravity (corr. SG) obtained
860 from wild (left) and zoo-housed (right) bonobos in relation to chronological age at sampling. Each
861 filled circle represents a sample: females in red, males in blue. Lines represent the fitted sigmoidal
862 model for the data set. Shaded areas represent bootstrapped 95% confidence intervals for expected
863 urinary neopterin levels. Total N = 806 urine samples, N_{wild} = 578 samples, $N_{zoo\text{-}housed}$ = 228 samples.

864 Figure 2: Log-transformed urinary neopterin levels corrected for specific gravity (corr. SG) obtained
865 from wild and zoo-housed female (red) and male (blue) bonobos in relation to chronological age at
866 sampling. Each filled circle and vertical black line at the buttom represents a sample. Lines represent
867 the fitted model for the data set. Shaded areas represent bootstrapped 95% confidence intervals for
868 expected urinary neopterin levels. Total N = 806 urine samples, N_{female} = 495 samples, N_{male} = 311
869 samples.

870 Figure 3: Estimated differences in age-related urinary neopterin levels for male and female bonobos.
871 Negative values represent higher neopterin levels in males and positive values represent higher
872 neopterin levels in females. Age periods with significantly different neopterin levels between the sexes
873 are indicated in red: Males have significantly higher neopterin levels between 0.4 - 2.7 years of age,
874 while females have significantly higher levels between 5.9 - 7.7 years of age. Total N = 806 urine
875 samples.

876

Figure 1.TIF

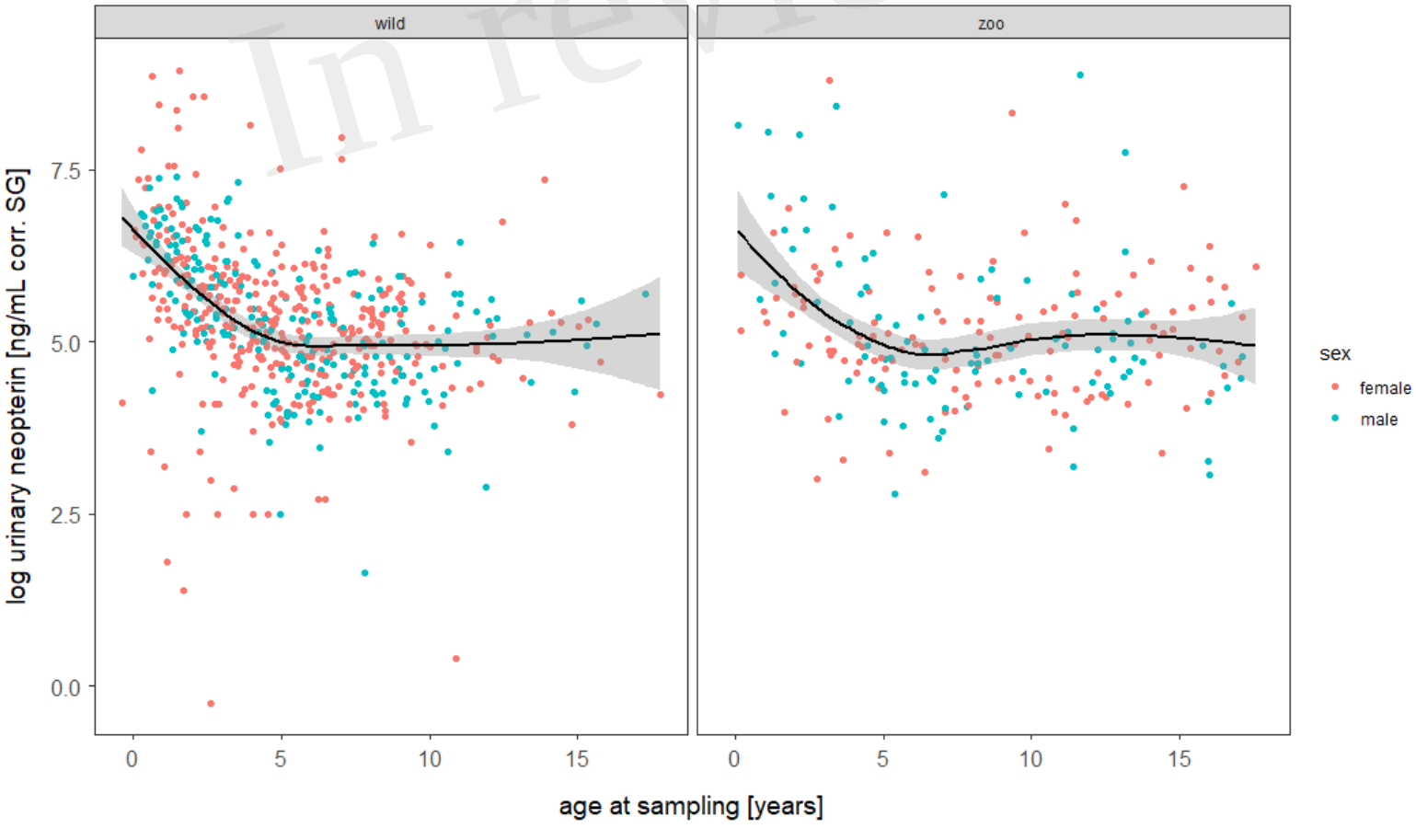


Figure 2.TIF

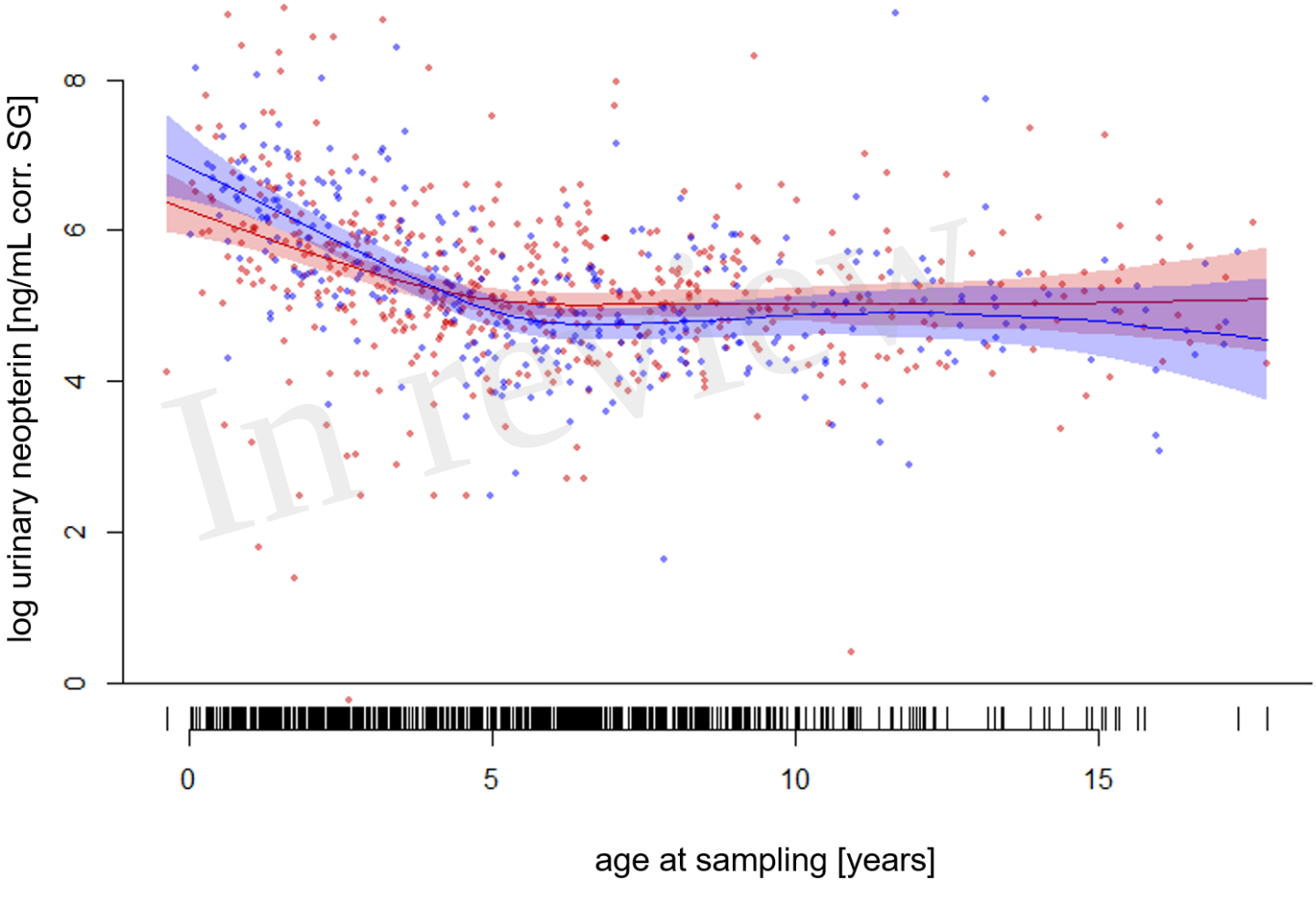
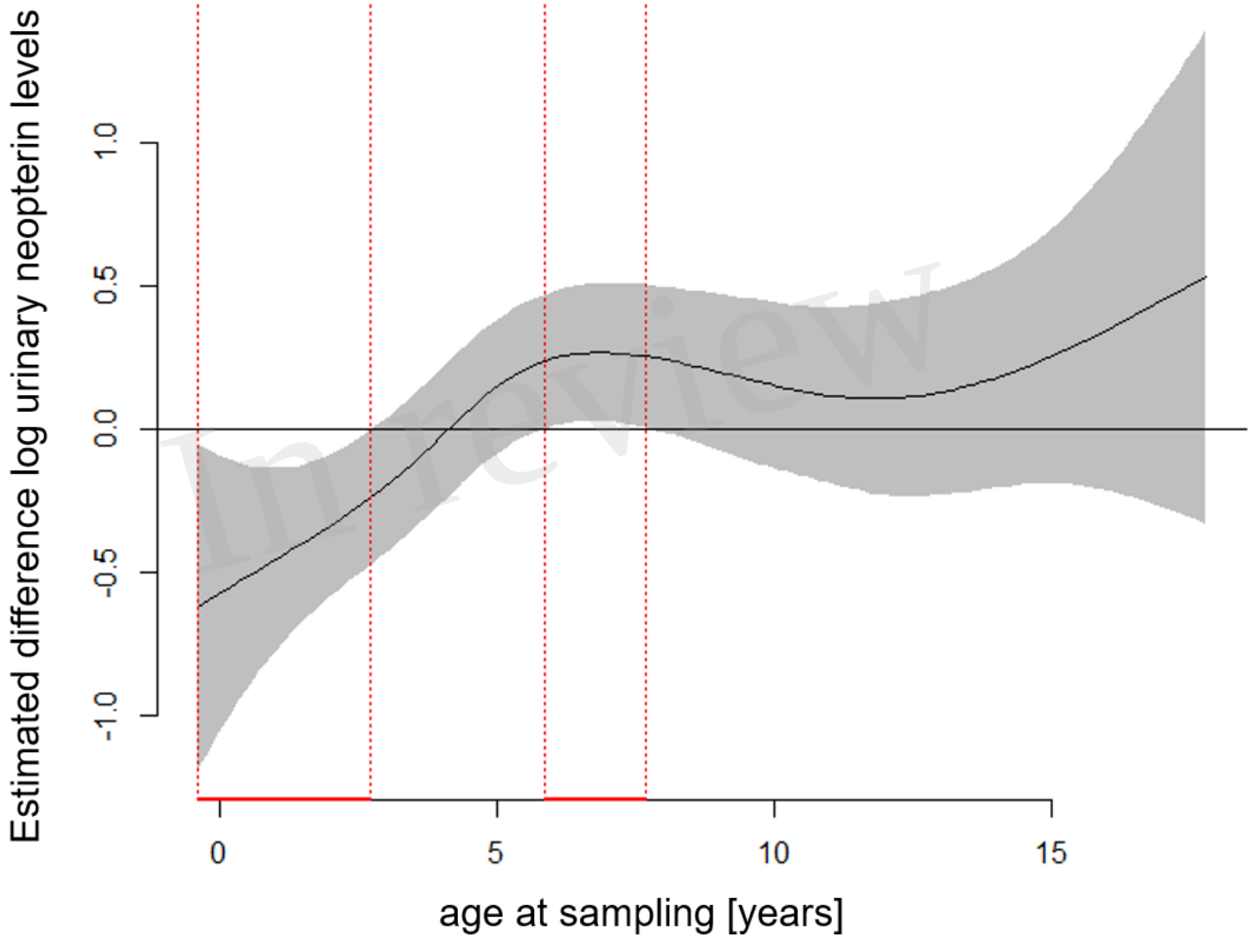


Figure 3.TIF


Estimated difference log urinary neopterin levels
1.0
0.5
0.0
-0.5
-1.0
0
5
10
15
age at sampling [years]