The influence of polyI:C during different stages of pregnancy on the development of psychiatric disorders in offspring

The variations in heart rate variability and ultrasonic vocalizations in infant rats.

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Abstract

Background
Psychiatric disorders are known for their high disease burden, even though it is still uncertain what exactly causes these disorders. However, it is known that environmental factors such as infections can largely influence the developing brain. Previous studies have shown that maternal infections during pregnancy increase the risk of psychiatric disorders in offspring, but many specifics still remain to be unravelled. It is important to gain more knowledge about this subject in order to improve treatment and prevention of psychiatric disorders.

Methods
In the current paper, the influence of polyI:C injections in pregnant rats on the development of psychiatric disorders in offspring is assessed. We compare two different stages of pregnancy; gestational day (GD) 9 - corresponding to the first trimester in humans - and GD15 - corresponding to the second trimester. The offspring is analysed based on heart rate variability (HRV) and ultrasonic vocalizations (USV) on postnatal days 7 and 14.

Results
The difference in bandwidth of USV's, as a measure for the complexity of USV's, showed a significant interaction between the treatment day of the pregnant rat and the sex of the offspring. Specifically, there was a difference between males and females for treatment at GD9, with a p-value of 0,004. Other than that, no significance was found in any variables in both the HRV and the USV measurements. However, non-significant correlations in mainly the USV data suggested a difference for the interaction of sex and treatment day. Possibly polyI:C at GD9 affects males more, while polyI:C at GD15 affects females more.

Conclusion
Possibly there are differences between male and female rat pups regarding HRV and USV's. No significance could be found in the current research to support this idea, but slight differences have been observed. Combined with previous research this does indicate that differences could be present. Further research is warranted to establish the relationship between the sex of the offspring and different moments of maternal immune activation during pregnancy.

1 INTRODUCTION

Mental and addictive disorders are known to have a high disease burden, both for individuals and for societies. In 2016, this resulted in a prevalence of these disorders of more than one billion people worldwide, leading to a loss of 162.5 million disease-adjusted life years (DALY's) worldwide in 2016. (Rehm & Shield, 2019) This substantial prevalence has multiple reasons, one of them is the stigma on these disorders, which results in a lack of treatment and a late start of treatments. On top of that, medicines to treat these diseases often don't have the desired effects. One of the best known examples is treatment resistant depression: 20-40% of the people with major depressive disorder (MDD) don't respond to the first treatment and only 50% of those people respond to the next antidepressant. After that, the chances that further treatment has clinically relevant effects are very slim. (Pandarakalam, 2018) Similarly, about 20-50% of schizophrenia patients are resistant against treatment and other psychiatric disorders show this phenomenon as well. (Nucifora et al., 2019)

Another reason for the high disease burden is that it can be challenging to diagnose these diseases, because questionnaires like the ICD-11 and the DSM-5 are used instead of objective parameters. Therefore, it can be complicated to diagnose young children with these illnesses, as they cannot fully express themselves yet. At the moment there is no other approved diagnostic tool for children. However, multiple studies have shown that an early diagnosis benefits the course of psychiatric diseases. (Rojas et al., 2019; Albert & Weibell, 2019; Rehm & Shield, 2019)

The statistics show that we need to understand more about the development of these disorders. Most of them are thought to be influenced by the same combinations of genes and abnormalities in the gene transcription process. That means they will most likely have common molecular routes of development and can thus can be influenced by the same factors. (Al-Haddad et al., 2019) It has also been observed that lots of similar brain areas are involved in a wide range of psychiatric diseases. (Goodkind et al., 2015)

But at the same time, one psychiatric disorder can be the result from a range of different gene combinations. That can also be the reason that treatment plans don't work for all patients, as not every patient for a specific disorder has the same underlying genetic profile. Moreover, the genetic profile isn't the only factor that can induce psychiatric disorders: external factors have a big influence as well. (Gaebel & Zielasek, 2015) There are lots of different external - or environmental - factors to think of, which can be categorized in pre- and postnatal factors. Things such as sickness or complications during pregnancy are prenatal factors and trauma or childhood stress are examples of postnatal factors. (Murray & Lewis, 1987)

These environmental factors have a great influence on psychiatric disorders, as is shown with 'twin experiments'. These showed that identical twins have around or less than 50% chance of both developing schizophrenia, despite their exact same genes. That means the other 50% is due to other factors, such as environmental factors. (Murray & Lewis, 1987)

The same kind of twin studies showed that 55% of autism spectrum disorder (ASD) is the result of environmental factors and only 37% of genetic factors. (Brown, 2012) These correlations have also been proven for MDD, bipolar disorder, alcoholism and substance abuse. (Ellenbroek & Youn, 2016) It's important to focus on the exact environmental factors that are causing these disorders, for a better understanding, diagnosis and treatment.

One of these factors is related to the immune system. It is known that infections could trigger the development of psychiatric disorders like post-traumatic stress disorder (PTSD), MDD, schizophrenia and anxiety disorders. This could be the result of the psychological impact of the disease or of the

infectious agent, but the immune response itself can have an important effect too. During infections, interleukins and interferons are released to activate the immune system, which induces cytokine dysregulations. This topic has enhanced relevance now, after the global pandemic of COVID-19. With the growth of urbanization, human population and climate change in mind, it is likely that epidemics and pandemics will occur more often in the near future. Hereby it is imperative to study the effects related to psychiatric disorders now. (Mazza et al., 2020; Gaebel & Zielasek, 2015; Estes &Mcallister, 2016)

1.1 INFECTIONS DURING PREGNANCY

Pregnant women are exposed to an added risk: when they suffer infections, it can have substantial consequences for both them and for their foetuses. (Cui et al., 2009) As most pathogens are unable to cross the placental barrier, these dangers for the foetus are most likely the result of maternal immune activation. (Robbins & Bakardijev, 2012) Likewise, autoimmune disorders, acute stress and external pollutants induce the maternal immune system and also present an enhanced risk of psychiatric disorders. (Cui et al., 2009) It is thought that an infection triggers the expression of major histocompatibility complex I (MHCI) molecules. While these are immune molecules, they also function as regulators for synapse formation. (Estes & Mcallister, 2016) Because development of the brain begins prenatally, complications during pregnancy are thought to have a significant influence on psychiatric disorders in offspring. (Cui et al., 2009)

One of the first studies on this topic was done after the 1957 type A2 influenza epidemic. This Finnish study found that exposure with this type of influenza during the second trimester of pregnancy gives an elevated risk of schizophrenia. (Mednick et al., 1988) In a response to that, lots of other studies have been conducted that study psychiatric disorders after maternal infections, but they gave contradicting results: A meta-analysis showed that a significant correlation exists between infections during pregnancy and ASD, but there was no significant difference in the prevalence of viral infections in the different trimesters of pregnancy. (Tioleco et al., 2021) But a Danish study found that there was no association between maternal infections and the diagnosis of ASD, except for when the pregnant women were admitted to the hospital because of the infections. In that case, there was only a correlation with ASD when women were infected in the first trimester of pregnancy. (Atladóttir et al., 2010) Contradictory, another study found that the severity of the infection does not influence the development of psychiatric disorders such as ASD. (Al-Haddad et al., 2019) There is just as much contradicting information in studies that focussed on other psychiatric disorders: one study found that the occurrence of affective psychosis was significantly increased after infections in the third semester of pregnancy, but the effects in the first and second trimester were not significant. (Blomström et al., 2016) However, a nested case control study found that there was a significantly induced risk of schizophrenia after influenza exposure during the first trimester of pregnancy, but the effect was insignificant in the second and third trimesters. (Brown et al., 2004)

Even though lots of studies have looked into the correlation between maternal infections and psychiatric diseases, lots of uncertainties remain. A relationship is highly suspected, but not yet proven. Most of the earlier studies only looked at pregnancies during epidemics as a marker for infections, which isn't accurate as it is unsure how many women were actually infected. Later studies mostly corrected for that by looking at individual infections. But still these studies have lots of variables: other complications during pregnancy, birth or after birth are largely unknown. Also, lots of later studies only look at hospital admissions as measurement of infections, even though women can also be infected without going to the hospital, or without even getting sick. This creates a bias in terms of underestimation. (Khandaker et al., 2013)

Furthermore, a difference in reporting infections and evaluating psychiatric disorders by different doctors and in different countries can give varying outcomes. Also, ethnicity, geography and development during childhood can affect the results. These factors are especially important because most studies have been conducted in Scandinavia and the USA, which means that the data is not representative for the rest of the world's population. (Haddad et al., 2020)

On top of that, there are a lot of studies that show an effect which isn't statistically significant. Most of those studies have relatively small sample sizes, which increases the amount of uncertainty. Possibly these results would have been significant when the sample sizes would have been larger. (Khandaker et al., 2013) Moreover, lots of different study designs, inclusion criteria's and socio-demographic differences make comparison of these studies with one another complex. All these variables cause a range of uncertainties: to date it isn't clear if the moment, the severity and the type of infections during pregnancy influence the development of psychiatric diseases in offspring. (Haddad et al., 2020)

1.2 ANIMAL STUDIES

To reduce the influence of the above mentioned variables, animal experiments can be conducted. With standardization of as many variables as possible and usage of highly controlled environments, more definite insights can be gained.

Previous research showed that rats that were administered a virus-like compound on day 15 of pregnancy got offspring with altered behaviour compared to placebo. They showed more avoidance and a lower ability for position discrimination. It is thought that the parahippocampal region of the brain develops at around gestational day (GD) 15 in rats. (Sarnat, 1992) It has also been seen that infections on GD 15 give an increase in the dopaminergic activity in various brain regions of the offspring. (Zuckerman, Lee et al., 2003; Meyer et al., 2006) Both of these observations can be correlated to psychiatric disorders. In addition, the use of clozapine and haloperidol as antipsychotic did give more 'normal' behaviour in the maternal infection group. (Zuckerman, L. et al., 2001; Zuckerman, Lee & Weiner, 2005) Another study in mice showed a significant increase in changes in the behaviour of adult offspring, when their mothers were infected with an influenza virus in the second trimester of pregnancy. In this study, the virus was artificially induced on day 9.5. (Shi et al., 2003) one more study in mice showed that immune activation on GD 9 lead to suppression in exploratory behaviour, latent inhibition disruption, reduced prepulse inhibition, enhanced sensitivity to amphetamine and dizocilpine and a deficiency in spatial working memory. But on GD 17, it gave retarded reversal learning, spatial working memory impairments and a response to amphetamine and dizocilpine. So some of these symptoms overlap, but some differ, indicating that different psychiatric diseases could have developed. This idea is supported by the observation that infections in different moments of pregnancy give different cytokines responses. (Meyer et al., 2009; Boksa, 2010)

Because these changes are mostly seen in adult offspring and not in infant offspring, some researchers hypothesize that infections mainly induce the development of schizophrenia in offspring, rather than other psychiatric disorders. That would make sense, as schizophrenia develops later in life in most individuals. (Häfner & Heiden an der, 1997) But there is sufficient evidence for the development of other psychiatric disorders, like depression, anxiety and ASD after maternal infection as well. This is mostly seen in behavioural studies that compare specific behaviour in rats with human behaviour. (Haddad et al., 2020) Those disorders are generally present from a young age, but behavioural differences are challenging to measure in rodent pups, as they barely move at such a young age. Moreover, it is still challenging to distinguish one psychiatric disease from the other in adult rats, because of the significant amount of overlap in both the presence and symptoms of these disorders. For instance, sensorimotor gating deficiency can be a symptom of both ASD and schizophrenia. (Haddad et al., 2020) It is unknown if psychiatric symptoms in humans are translatable to rodents and

thus, it is detrimental to talk about certain disease types with regard to rodent studies. (Gass & Wotjak, 2013)

Therefore, the rest of this paper will focus on the occurrence of psychiatric disorders altogether. If there is a specific moment in pregnancy in which the risk on all of the disorders is the highest, this is valuable information to know. The rat brain develops mostly during the second trimester, so we hypothesize that infections during late pregnancy in rats will give a greater chance of psychiatric disorders. (Pressler & Auvin, 2013; Haddad et al., 2020)
We also hypothesize that there is a correlation between the moment of infection during pregnancy and the development of psychiatric disorders in the offspring, which is dependent on the part of the foetus that is developed at that moment. (Haddad et al., 2020)

1.3 EXPERIMENT

In this experiment we will study the effects of virus-like, maternal immune activation at two different stages of pregnancy on developing psychiatric disorders in offspring in rats.

For that, two different gestational days will be compared: day 9 and day 15. These roughly represent the first and second trimesters of human pregnancy. It is difficult to extrapolate the human pregnancy trimesters to rats, but it is known that rats are born with a less developed brain than humans. The rat's brain will be similarly developed to that of newborn human children at postnatal day 7-13. That means that the first half of the pregnancy in rats roughly resembles the first trimester in humans, the second half of pregnancy in rats resembles the second trimester in humans, and postnatal day (PND) 0 to 7 in rats resembles the third trimester in humans. (Boksa, 2010) Therefore we will make a distinction between the first and the second trimester of pregnancy. Day 9 and 15 were specifically chosen because these days appear the most in previous research, which simplifies comparison.

The pups will be analysed on PND7 and 14. As mentioned above, days 0 to 7 resemble the third trimester in humans, so day 7 can be compared to the birth of the human child. (Boksa, 2010) We will measure ultrasonic vocalizations, which are the strongest around day 7, so differences between the groups will be easiest to measure on that day. Also, it has been studied that the cerebral cortex of rats is developed to the same extent as that of human neonates at postnatal day 13. (Romijn et al., 1991) However, PND14 is preferred, because most previous research has tested at that day. By choosing the same test day, it will be easier to compare the results. Assuming the 1 day difference won't give significant alterations in the outcomes, we can still compare the results with the situation in newborn children.

1.3.1 PolyI:C

During the experiment, we won't use an actual virus. Instead, we will use a virus-like compound that simulates a general viral reaction in the body. That means we don't have to make a distinction between different viruses and we will get a more general result.

The compound that we will be using is called polyinosinic:polycytidylic acid (polyI:C), which is widely accepted to simulate viral infections. It is a synthetic analog of double stranded-RNA that can be found in replication cycles of dsRNA viruses, single stranded RNA viruses and double DNA viruses. It can activate human toll-like receptor 3 (TLR3), which activates a downstream signalling pathway that stimulates the production and release of a range of pro-inflammatory compounds, such as TNF-α, IL-6 and IL-1β. These compounds can travel through the bloodstream to trigger cellular and molecular changes in a variety of cell types, like neurons. On top of that, the release of type 1 interferons like IFN-α and IFN-β are stimulated and the compound can induce fever for up to four hours. The activation of TLR3 differs from the activation of other TLR subtypes, because each subtype activates a slightly different downstream cascade and each subtype is expressed to a different extent in organs. It is

known that TLR3 is present in many organs, but it has an especially high expression in the placenta, indicating that it could influence the foetus more. Because a viral infection gives similar effects, polyI:C mimics the acute phase response of such an infection. (Boksa, 2010; Meyer et al., 2009c; Haddad et al, 2020)

Usage of polyI:C means no specific antibodies will be formed because no virus particles are present. Therefore we can be certain that the effects are the result of solely the maternal immune activation, rather than a combination with the consequences of a specific virus. An advantage of using polyI:C instead of a virus is that it will give immune reactions only for a short period of time (approximately 48 hours). That means that the reactions in offspring can be related to a very specific time frame during pregnancy. This simplifies the comparison between the two different moments of infection that we are going to study. (Zuckerman & Weiner, 2005) Also, the composition of the injection is exactly known, so it is known how much is injected. There is no risk of a multiplying virus that could activate the immune system even further. Therefore, using polyI:C takes away lots of variabilities that real viruses present. (Cui et al., 2009)

1.3.2   Outcomes

Most studies in this area exclusively look at behavioural changes of rodents, which can be measured from the age of a month. But it is known that an early detection of these diseases can improve the treatment plan from a young age, which results in a reduced severity of the disease over the course of life. (Albert & Weibell, 2019) Therefore, we want to look for parameters that can be measured as early in life as possible. Most behavioural differences are only measurable from an older age, because young pups are not very active and move barely. Hence, heart rate variability (HRV) and ultrasonic vocalizations (USV's) will be used in this experiment. These can be measured from a very young age and they have shown to have a relationship with psychiatric diseases, which in turn is helpful for an early treatment. (Albert & Weibell, 2019) Also, it is very easy to combine the two outcomes in one measurement, because the pups will separated from their mothers to measure the HRV. At separation, infant rats are known to produce USV's, which can directly be measured with a microphone. As USV's are the only behavioural signs that can be measured in such young pups, this is also a way in which cardiac outcomes can be related to behavioural changes, making it appropriate to look at the combination of these two outcomes.

1.3.3   Heart rate variability

It is known that most psychiatric disorders have a lot of comorbidities, especially in the cardiovascular system. This indicates that the two disease types are correlated to each other. It is still very difficult to objectively measure most brain processes (Thapa et al., 2019), but the correlation with the cardiovascular system can help. The heart rate is controlled by both the sympathetic and the parasympathetic nervous system, via the stellar ganglion and the vagus nervus respectively. That means that the HRV can function as an indicator for how well these systems work. This measure is characterized by the standard deviation between the R-R peaks during an ECG measurement. This represents the depolarization and thus the contraction of the heart chambers, so by comparing the intervals between these peaks, the HRV can be studied. (Haigh et al., 2021) A lack of heart rate variability indicates a lack of flexibility in the brain, with an elevated sympathetic activity and/or a decreased parasympathetic activity. This is indicative of autonomic dysfunction, as those are also influences by both the sympathetic and the parasympathetic nervous systems. Hence, the HRV can serve as an indicator for psychiatric diseases. Specifically, stress results in a hyperactivation of the sympathetic system with a reduction of the HRV. The bigger the parasympathetic influence still is, the higher the HRV will be and the better the individual will be at dealing with stressful situations. (Park et al., 2017) As it has been proven to correlate to psychiatric disorders too, it is now widely accepted to use HRV as a predictor for these disorders. (Thapa et al., 2019; Carnevali et al., 2017; Servant et al., 2009)

The measurement of the HRV in humans is very similar to that in rats and that increases the potential for interpolation. (Cygankiewicz & Zareba, 2013) Even though this phenomenon isn't as extensively studied in rodents as in humans, correlations have been shown in rodent models as well. Multiple studies have recently been conducted that indicate a relationship between the HRV and the presence of psychiatric disorders in rats. (Carnevali et al., 2017; Sgoifo et al., 2015) Both rats and mice also show a reduced heart rate variability in stressful states, indicating that this parameter is indicative of the emotional state of rodents as well as humans. (Liu et al., 2013)

For the data analysis, 10 seconds of measurement are sufficient to obtain reliable numbers, however a 15 second interval is preferred. But the heart rate is highly variable and can be influenced by short-term stress, amongst others. (Tiwari et al., 2021) To rule out these variances as much as possible, a 8-minute measurement per rat will be made, after which as much intervals as possible will be analysed. 10 to 15 second intervals will be used, depending on the amount of intervals that can be generated for every length. we will only look at linear parameters of HRV. Even though they don't account for the influence of other systems in the body than the heart, they are well understood and the most used. One of these parameters is the root mean square of successive heartbeat interval differences (RMSSD). This is a statistical measure which mainly looks at the influence of the parasympathetic nervous system on the HRV. We prefer this parameter over the standard differentiation of the R-R intervals because the latter is influenced by the duration of the data. We use varying interval durations to obtain the most data as possible, so R-R intervals would be unreliable. In this situation, the RMSSD can be used. This parameter can be obtained by tracking the root mean square of the intervals that are obtained after performing a fast Fourier transformation. However, it is thought that the sympathetic nervous system also influences this parameter. To be able to differentiate between the two, other outcome measures will be used. We will include the high frequency power band (HF), the low frequency power band (LF) and the low frequency/high frequency ratio (LF/HF). HF stands for the parasympathetic activity. It represents the activity of the vagal tone by reflecting the variation in heart rate during the respiratory cycle. LF stands for a mix of parasympathetic and sympathetic activity, but it is more sensitive for the sympathetic system. We will also look at the relation that the two systems have with each other with the LF/HF ratio. (Kidwell & Ellenbroek 2018)
RMSSD and HF are considered reliable predictors for parasympathetic activation of the heart, but less evidence exist for the LF and the LF/HF ratio as predictors for the sympathetic activity. These parameters seem to be influenced by both the sympathetic and the parasympathetic system. (Schiweck et al., 2019) Therefore it is not known how accurate these parameters are and we will focus more on the high frequency, together with the RMSSD.

A systematic review in humans showed lower HF intervals in individuals with MDD in 6 out of 8 studies they looked at. However, the difference was mostly insignificant. (Schiweck et al., 2019) Another meta-analysis found that people with varying anxiety disorders have a lower HRV than controls. The largest effects were observed in people with generalized anxiety disorder and PTSD. Specifically, lower RMSSD and HF values were found, but the LF/HF was not affected by anxiety disorders. (Cheng et al., 2022) Even though these studies are all performed in humans, we expect a similar effects in rats. As another study already demonstrated, symptoms of anxiety disorders in rats are also correlated to lower RMSSD and HF values and higher LF values. This study didn't find significant differences in the LF/HF values either. (Carnevali et al., 2014) We therefore hypothesize that the HRV will be decreased in rats with a maternal polyI:C treatment compared to saline treatment, which is expressed in lower RMSSD and HF values than the controls.

1.3.4 Ultrasonic vocalizations

The USV's that are produced by rodents are used for social communication between individuals. USV's for different purposes can be distinguished; from parenting, to anxiety, playing, aggression and the presence of danger. It has been shown that rats produce more of these vocalizations than mice, which makes rats more interesting for research in this topic. (Caruso et al., 2020)

As infant rats get isolated from their mothers, they will produce ultrasonic vocalizations.

These isolation-induced calls occur at 40 kHz on average, with a range of 30 to 60 kHz, and normally stimulate the mother's caregiving. (Jouda et al., 2019) Changes in the rate of these vocalizations correspond to the amount of anxiety that young rats of 3-18 days old experience. (Lenell et al., 2021) After those 18 days, the amount of these specific calls will decrease fast, because the rats will be more independent by then. (Hofer, 1996; Morales-Navas et al., 2020) Several studies have demonstrated that an increase of these infant USV's are correlated to adult behaviours that resemble anxiety and depression. (Simola et al., 2018; Lenell et al., 2021) Young children that later develop psychiatric disorders show differences in crying too, with abnormal patterns and higher frequencies. (Morales-Navas et al., 2020) Because USV's are correlated with social behaviour and mental illnesses are diagnosed according to behaviour, it can form a good early diagnostic for rat experiments. These vocalizations can function as indication for a range of psychiatric diseases with reduced social behaviour, like ASD, schizophrenia, addiction and depression. (Simola et al., 2018)

Lots of experiments have been conducted that look at ultrasonic vocalizations in infant rats as an early predictor for psychiatric disorders. Because correlations have already been seen, this outcome will be used to compare the heart rate variability with. That is a promising, but more unknown outcome for rat experiments and thus, we will look at the differences in HRV and USV's in offspring of infected and uninfected rats.

After measurement of the ultrasonic vocalizations, multiple parameters can be used for further analysis. In this study we will focus on the total amount of calls and the total duration of the calls. But it's also possible to look at differences within the calls, like the bandwidth. This represents the difference between the highest and lowest frequency within the call and gives information about the complexity of them. This is thought to vary too, not only with regard to the treatment of the mother, but also over time and between males and females. Furthermore, we will look at the amount of harmonic calls. These are defined by two calls at the same time and in the same shape, but one call has twice the frequency as the other one.

Previous research has found that prenatal polyI:C injection on GD12,5 gives a decrease in the number of calls, but there was no change in the acoustic characteristics of the calls. Furthermore, this study showed that males emit significantly more calls than females. (Potasiewicz et al., 2020) There is limited data about the influence of different moments of the polyI:C injection, but most studies that looked at GD15 have found a significant decrease in USV's, which means that less anxiety is present upon short maternal separation. (Chou et al., 2015) That decreases the chances of survival, as the mother will be less likely to retrieve the pups upon separation when they have less USV's. (Brunelli et al., 2015) At GD 15, cortical neurogenesis and development of major fibres take place. In humans, this process also happens in the second trimester. (Chou et al., 2015; Haddad et al., 2020) Therefore, we hypothesize that exposure to polyI:C will have a greater effect towards late pregnancy. In line with that idea, injections on GD9 will have a lesser effect on USV's than injections on GD15. But in both cases, we expect a decrease in both the number and the duration of the calls, while the bandwidth is expected to be similar.

The sex differences are also important with regard to USV's. Males are known to produce more USV's that are less complex than females. That means the mother is more likely to hear male pups, which stimulates male survival over female survival. (Lenell et al., 2021)

2 METHOD

2.1 EXPERIMENT

20 female and 20 male Sprague Dawley rats will be bred at the Te Toki a Rata vivarium at the Victoria University of Wellington. They will be housed in groups of 2 to 4 animals in housing facilities on a reversed 12-hour day/night cycle. The lights turn off at 07:00 and they turn on at 19:00 every day. The rooms will have a controlled temperature of 20 degrees Celsius with a 2 degree variability. The animals have unlimited access to water and food. The animals will not be disturbed apart from the actions necessary for the experiment and a weekly cleaning of the cages.

On the morning of the first day, every female rat will be combined with a male rat to share a cage with a grate with small holes on the bottom. Every morning and every afternoon the rats will be checked for pregnancy. This will be determined by looking for a vaginal plug, which female rats drop when intercourse has occurred. They will then drop through the grate, which prevents the rats from eating it. The plug doesn't guarantee that fertilization has happened, instead there is about 92% chance that it did. (Voipio & Nevalainen, 1998) Therefore, for the ease of the experiment we will assume that fertilization happened when a plug is present and that will be labelled prenatal day 0. At that moment, the male and female rats will be separated, after which the females all have a separate cage with normal bedding and the males will be combined in groups of 2 to 4 rats.

The 20 female rats will be randomly assigned into four different groups:
1. Injection with polyI:C 2 mg/kg on day 9 of pregnancy;
2. Injection with polyI:C 2 mg/kg on day 15 of pregnancy;
3. Injection with saline 0,9% on day 9 of pregnancy;
4. Injection with saline 0,9% on day 15 of pregnancy.

Once the female rats are pregnant, they will be administered either polyI:C 2 mg/kg or a saline equivalent subcutaneously on either GD9 or GD15, depending on the group that they are in. The injection will always be given between 10:00 and 12:00. The body weight of each female rat will be determined before injection and 24 hours after injection. The body temperature will be determined before injection and 2, 4 and 24 hours after injection. From GD17, nesting material will be placed in all of the cages. From that day, each cage will be checked on pups twice a day. The moment of birth of the offspring will be considered PND 1. The litters will be left alone until PND7. At that day, two males and two females per litter will be used. Their sex will be determined by anogenital distance.

On PND7 and PND14, the rats will be studied on HRV and USV for approximately 8 minutes. These two measurements will happen at the same time. The heart rate variability will be studied with an ECGenie and with the computer program LabChart version 8.1.24. Before starting the measurement, the ground plate of the ECGenie will be heated, so the pups won't be cold and therefore will be less uncomfortable during the measurements. That will decrease the amount of movement, but it is also important for the outcomes: a drop in heat can increase the number of USV's. (Lenell et al., 2021) Conductive gel and copper tape will be used to increase the connectivity of the ECGenie.
Ultrasonic vocalizations will be studied by placing a microphone (Ultramic 250K) above the rat when they are in the ECGenie. The program Audacity version 3.2.1 will be used for data collection.

The measurements will always take place in the mornings (between 09:00 and 13:00) to reduce variance due to the time of the day. Because the rats all live in a reversed day-night cycle, the measurements will take place under red light to still create a dark environment.

Before starting the experiments each day, a test pup will always be used to test the equipment. In case of weak or non-existent signals, the pup will be removed from the setup and adjustments will be made if necessary. By using a testpup, we will decrease the likelihood of a failed measurement in the rest of the pups. Each pup can be separated from the rest of the litter for a maximum of 15 minutes. When separated any longer, the likelihood of adjusted behaviour like increased licking behaviour by the mother could increase and that could influence later measurements. (Azevedo et al., 2010)

After the PND7 measurements, each pup will be marked so it can be recognised for the PND14 measurements. After that day, the pups have to be remarked every day to avoid fading of the mark due to the mother's grooming. At the end of the day 14 experiments, all rats will be either euthanized or used for other experiments.

2.2 ANALYSIS

Labchart 8.1.24 will be used for the HRV data analysis. The intervals between the R-peaks in the electrocardiogram (ECG) will be assessed with a datapad. This will generate a fast fourier transformation of the data, to be able to generate the frequency outcome measures. During the data analysis of these measurements, we will search for usable intervals. Because the pups will only be restrained by a metal plate on top of the ECGenie, they will still be able to move slightly, e.g. lifting up their paws and thereby making a measurement impossible. Especially during the PND14 measurements, the pups will ruin a sufficient part of the measurement by moving. Therefore we will aim for 10 to 15 second intervals of usable data. The exact duration will be determined by the amount of intervals that can be generated for every duration. That decision will be made for each measurement individually. By adjusting the settings slightly, we hope to get the maximum amount of usable data for each pup.

After the intervals are obtained, they will be selected on suitability. The pRRx should be equal to zero, the RMSSD and the SDSD should have similar numbers for each interval and both numbers should be below 3,0. When the average RR of the first few intervals is lower than the average for that measurement, they can be deleted, as the pups have to get used to the ECGenie at the beginning of the measurement. And last, all values in the SD1 and SD2 columns should be similar to each other. When the numbers of intervals do not comply with these requirements, they will be deleted. From the remaining intervals, the medians of the RMSSD, HF power (%), LF power (%) and LF/HF will be calculated and those will be used for the statistical analysis. If necessary for a normal distribution, the numbers will be transformed into logarithmic values.

The USV's will be analysed using Raven Pro version 1.6.4. Because this program is originally designed for bird calls and the microphone we will use is fairly old, a lot of noise is expected in the measurements. To make sure that all calls are correctly located and noise is correctly ignored, all measurements will be analysed manually. The measurements last for about 8 minutes, so to ensure that the exact same duration can be used for all measurements, we will only analyse the first 7 minutes of each measurement. The duration of a single call should be at least 20 milliseconds and we will aim for a frequency of at least 30 kHz. In the case a call has a great bandwidth and only partly fulfils these requirements, every single call will be assessed individually. When part of the call is at least 30 kHz, but part of it drops to about 25 kHz, it will still be used for further analysis. When the call drops to lower than that, it won't be used. After selecting all suitable calls, the program automatically generates the data we need. The only thing that can't be analysed with this program, are the number of harmonic

calls. Therefore, these will be counted manually. In the end, the total number of calls, total duration of the calls, average bandwidth and the percentage of calls with harmonic calls will be used for the statistical analysis. If necessary for a normal distribution, the numbers will be transformed into logarithmic values.

For the statistical analysis, the medians for the intervals of all variables will be calculated per rat. Then, the means over the multiple rats will be calculated for every treatment group. Males and females will be separated too, to be able to make a distinction between the sexes. The statistical analysis will be executed in Jamovi Statistical software, version 3.2.21. For the total duration of calls, a negative binominal regression model will be used and for all other variables, a mixed model linear regression will be used. For the harmonic calls, an arcsine transformation will be used prior to the analysis with the mixed model linear regression. Normality will be tested first, with both a Q-Q plot and with a residuals-predicted plot. P-values will be generated with a fixed effects Omnibus test and values lower than 0,05 will considered to be significant. In case of significance, Bonferroni post-hoc tests will be executed.

3 RESULTS

The temperature and weight of the rats is displayed in Appendix A. it can be observed that the temperature did rise for a couple of hours after polyI:C injection, but not after saline injection. This supports the literature, stating that a short-term fever reaction can be induced because of polyI:C. (Haddad et al, 2020)

During the experiment, 5 adult females had to be excluded from the experiment for varying reasons. Three rats had complications during pregnancy, 2 rats turned out not to be pregnant and 1 rat had to be euthanized halfway through the pregnancy. For specific details, we refer to Appendix B.
Also, there was one rat that didn't show a plug for 1,5 weeks, after which she was separated from the male. Eventually she did gave birth to a healthy litter, so the plug was either missed or eaten by one of the rats. That rat was not injected, but the pups were still analysed to assess if the outcomes were similar to those of the saline rats. After data analysis, it turned out that for most variables there was no significant difference between the saline groups and the rat that didn't get an injection. But for the call duration, there was a significant difference for the interaction between the treatment and the sex. Therefore it was decided to keep the four groups separate for all of the variables. That did mean that the rat with no injection could not be included in the analysis.

After analysis, a few more separate measurements had to be excluded. For the HRV data, there were three measurements that could not be analysed due to a lack of usable intervals. After the data analysis, three more measurements had to be excluded due to high RMSSD numbers.
For the USV data, something went wrong with the first day's measurements and for almost all PND7 measurements for cohort 2. That means there is limited data for PND7, while the measurements for PND14 were mostly usable. However, there was 1 measurement that was only 6,5 minutes long. That one was excluded as well, because it can't be reliably compared to the rest. An overview over the total amount of used rats in each group can be found in Appendix B. For the means of every treatment group, see Appendix C. Appendices D and E can be used for the outcomes of the statistical analysis per variable.

3.1 HRV RESULTS
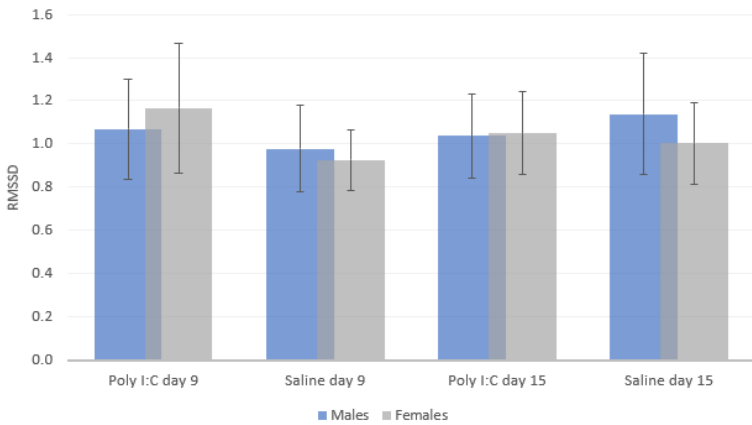
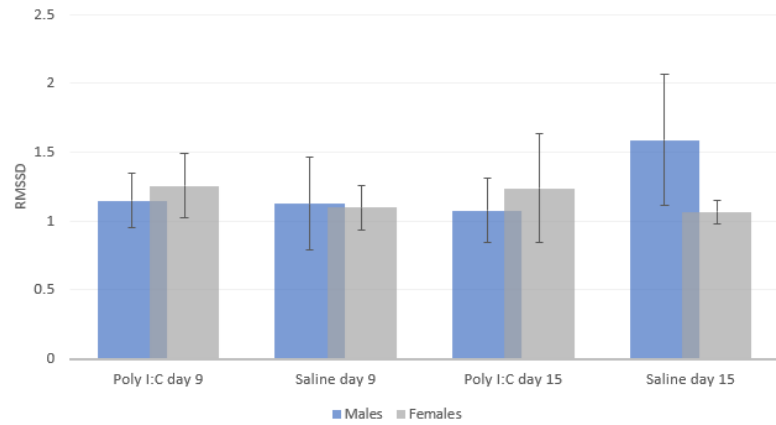
Figure 1a: The means ± SD of the RMSSD values measured at PND7 for the groups polyI:C day 9, saline day 9, polyI:C day 15 and saline day 15, in which males and females are separated.

Figure 1b: The means ± SD of the RMSSD values measured at PND14 for the groups polyI:C day 9, saline day 9, polyI:C day 15 and saline day 15, in which males and females are separated.

Figure 1a shows the means of the RMSSD values that were measured for each of the treatment groups at PND7. Figure 1b is identical, but displays the PND14 measurements. Significance was found for sex * treatment (p = 0,012) in the linear regression. That suggests that the influence of the treatment differs between the males and females. The graphs shows that the means of both polyI:C groups show a larger increase in RMSSD in females than in males. This is true for the PND7 measurements, with a mean of 1,069 (± 0,23) for the male polyI:C day 9 group, against 1,166 (± 0,30) for the female polyI:C day 9 group. For the treatment day 15 groups, the means were 1,037 (± 0,19) and 1,050 (± 0,19) for males and females respectively.

In the PND14 measurements, the treatment day 9 groups showed some difference, with means of 1,149 (±0,19) for males and 1,257 (± 0,23) for females. Males and females were more different in the treatment day 15 group than at PND7, with means of 1,078 (± 0,23) for males and 1,238 (± 0,40) for females. The RMSSD generally is higher in males in the saline group, whereas it's higher in females in both the polyI:C groups. However, no significance was found in the post-hoc test. Significance in the linear regression was also observed for treatment * treatment day (p = 0,035), which indicates that not only the difference between polyI:C and saline, but also the difference between administration days 9 and 15 is significant. But here, no significance could be found in the post-hoc test either.

Figure 2a: The Mean HF ± SD in percentages at PND7 for the groups polyI:C day 9, saline day 9, polyI:C day 15 and saline day 15, in which males and females are separated.

Figure 2b: The mean HF ± SD in percentages at PND14 for the groups polyI:C day 9, saline day 9, polyI:C day 15 and saline day 15, in which males and females are separated.

In Figures 2a and 2b, the mean percentual HF values can be seen for each of the treatment groups, for PND7 and PND14 respectively. There are no significant differences for sex (p = 0,626), nor for the treatment groups (p = 0,441).
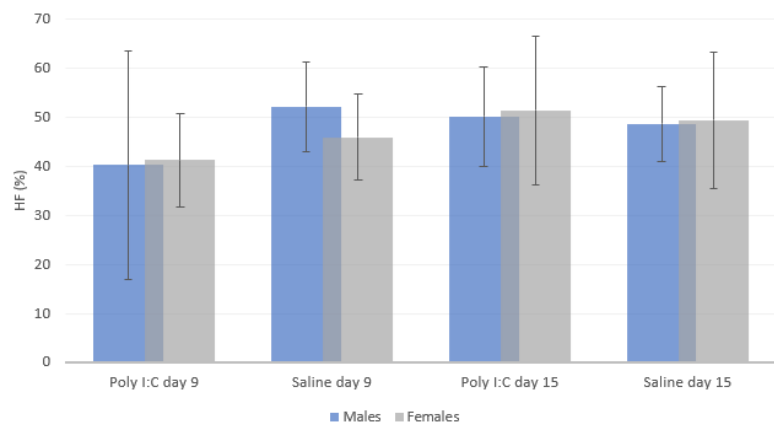

Figure 3a: The Mean LF ± SD in percentages at PND7 for the groups polyI:C day 9, saline day 9, polyI:C day 15 and saline day 15, in which males and females are separated.
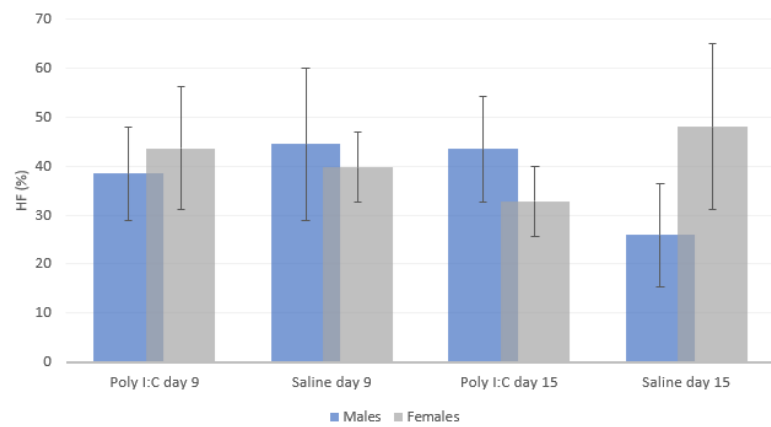
Figure 3b: The Mean LF ± SD in percentages at PND14 for the groups polyI:C day 9, saline day 9, polyI:C day 15 and saline day 15, in which males and females are separated.

Figure 3a shows the mean percentual LF for all treatment groups on PND7, and Figure 3b shows the percentual LF for all groups on PND14. Again, none of the differences showed any significance, with a p-value of 0,433 for the sex and a p-value of 0,084 for treatment * treatment day.
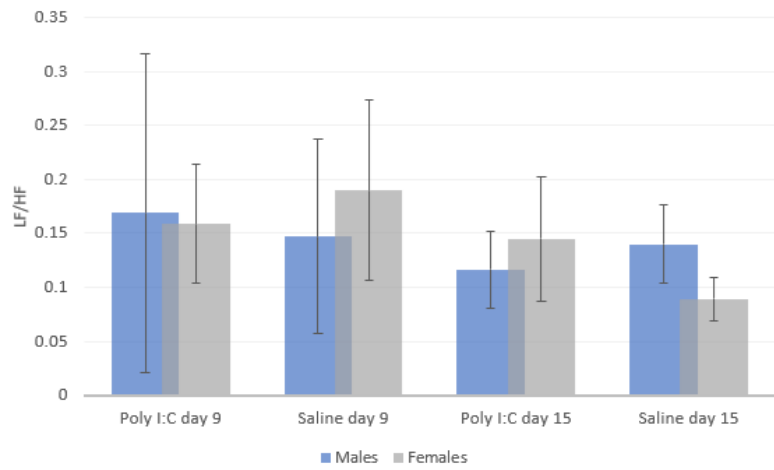
Figure 4a: The Mean LF/HF ± SD in percentages at PND7 for the groups polyI:C day 9, saline day 9, polyI:C day 15 and saline day 15, in which males and females are separated.

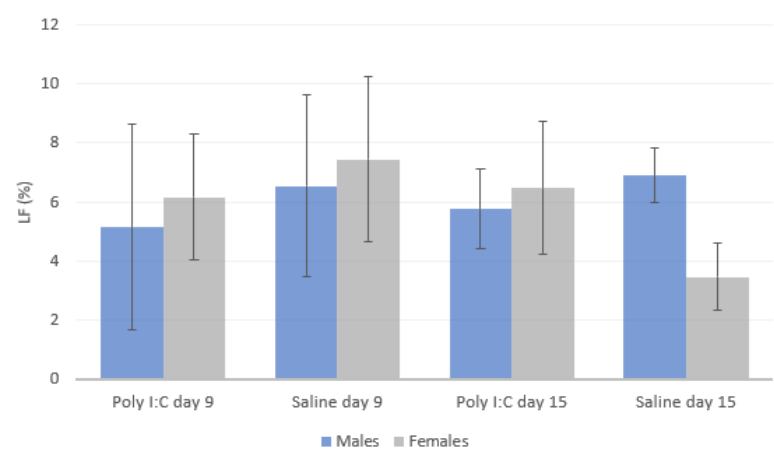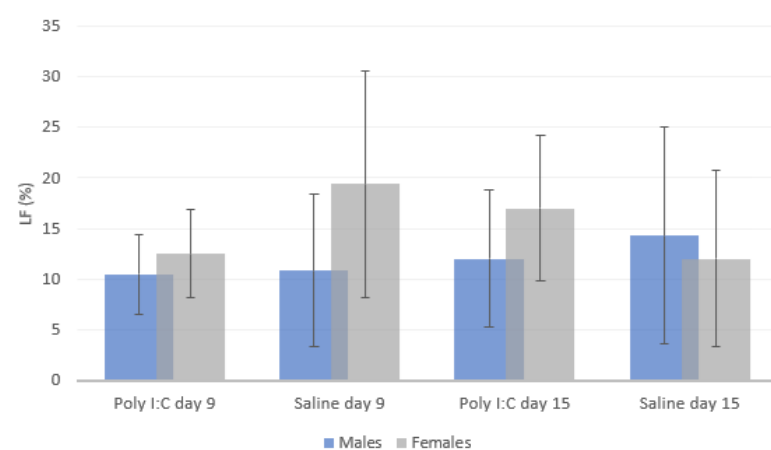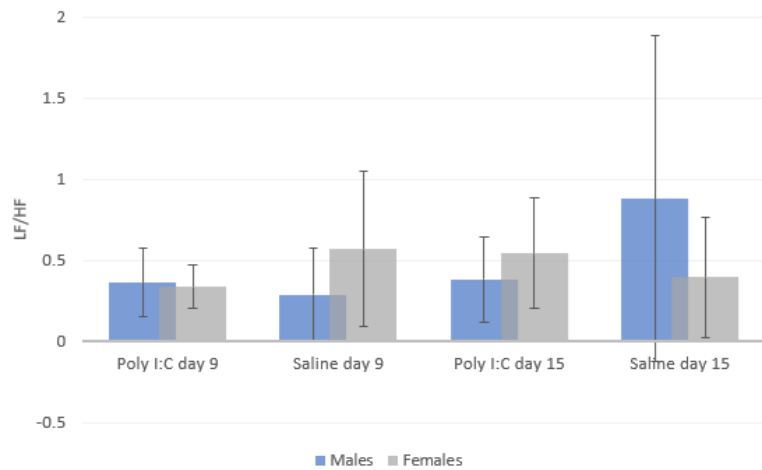*Figure 3b: The Mean LF/HF ± SD in percentages at PND14 for the groups polyI:C day 9, saline day 9, polyI:C day 15 and saline day 15, in which males and females are separated.*

In Figure 4a and 4b, the LF/HF ratio is shown for all treatment groups for PND7 and PND14 respectively. The p-value for sex is 0,704 and the p-value for treatment * treatment day is 0,390, indicating that there is no significant difference. None of the other variables proved to be significant.

3.2 USV RESULTS

Figure 5a: The mean of the total number of calls ± SD at PND7 for the treatment groups polyI:C day 9, polyI:C day 15, saline day 9 and saline day 15. Males and females are separately shown.

Figure 5b: The mean of the total number of calls ± SD at PND14 for the treatment groups polyI:C day 9, polyI:C day 15, saline day 9 and saline day 15. Males and females are separately shown.

Figeures 5a and 5b show the differences between the total number of calls between males and females and for all of the four treatment groups. These differences were not significant, with p-values of 0,773 and 0,865 for sex and treatment * treatment day respectively. However, the graph does show a slight relationship between a treatment of polyI:C and a reduce in the total number of calls at PND7. PolyI:C at GD9 seems to influence males more, whereas administration on GD15 seems to influence females more. No obvious correlation can be seen in the PND14 measurements.

Figure 6a: The mean call duration ± SD at PND7 for the treatment groups polyI:C day 9, polyI:C day 15, saline day 9 and saline day 15. Males and females are separately shown.
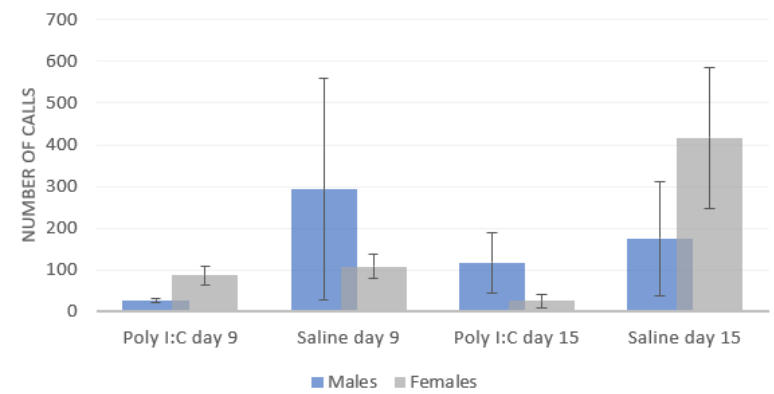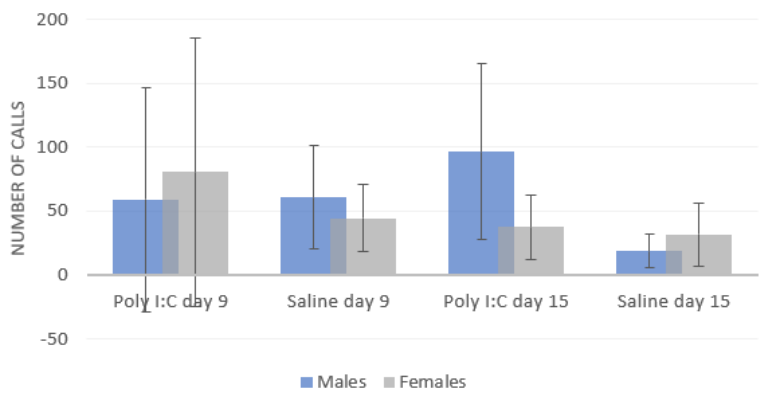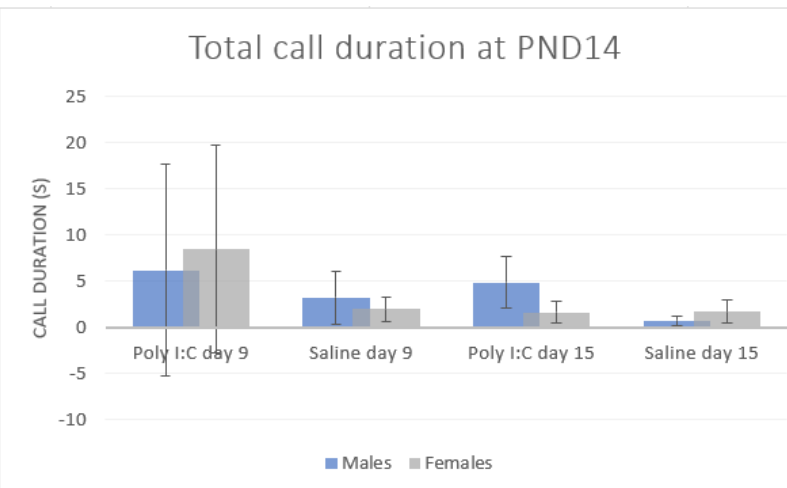
Figure 6b: The mean call duration ± SD at PND14 for the treatment groups polyI:C day 9, polyI:C day 15, saline day 9 and saline day 15. Males and females are separately shown.

Figure 6a shows the means of the total call duration at PND7, while Figure 6b shows the same for PND14. The p-values for sex (p = 0,895) and treatment * treatment day (p = 0,495) were not significant. In line with measurements of the number of calls, the total call duration in the PND7 polyI:C groups are both smaller than for the saline groups. Again, males seem to be more affected by prenatal polyI:C treatment on GD9, while females seem to be more affected by injections at GD15. The call duration appears to be higher in the male polyI:C groups on PND14, than their saline equivalents. For females, this is only true for the polyI:C day 9 group. These differences were not significant.
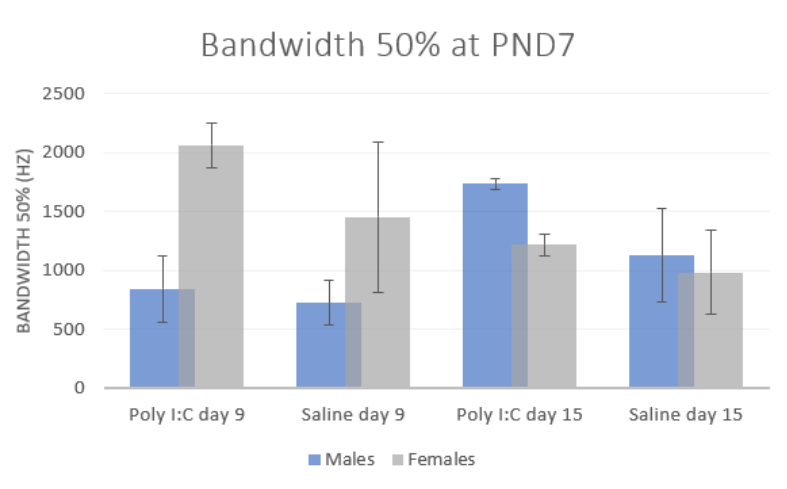
Figure 7a: The mean 50% bandwidth ± SD at PND7 for the treatment groups polyI:C day 9, polyI:C day 15, saline day 9 and saline day 15. Males and females are separately shown.
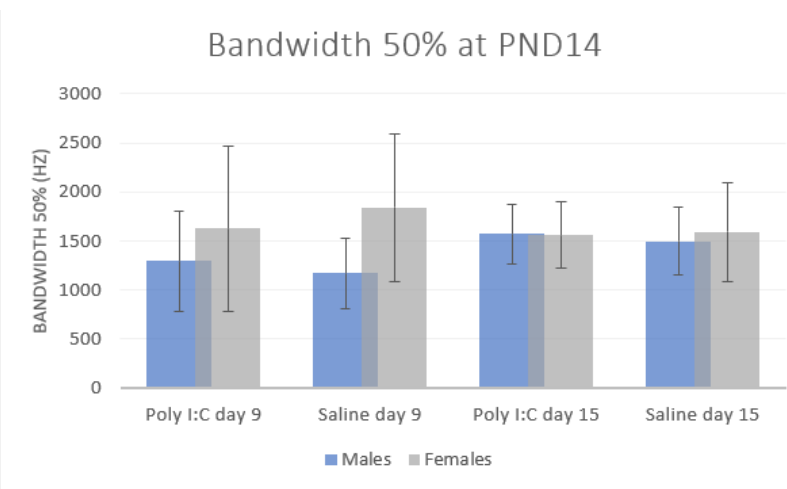
Figure 7b: The mean 50% bandwidth ± SD at PND14 for the treatment groups polyI:C day 9, polyI:C day 15, saline day 9 and saline day 15. Males and females are separately shown.

Figures 7a and 7b show the mean 50% bandwidth for all treatment groups at PND7 and PND14, respectively. A significant difference was found for sex * treatment day, indicating that there was a correlation between the sex of the animal and the moment that polyI:C was injected during the pregnancy. The post hoc test indicated that only the difference between male day 9 and female day 9 was significant (p = 0,004). As can be seen in Figure 7a, the bandwidth in males in the polyI:C day 9 group is about half of the bandwidth in males in the polyI:C day 15 group, with respective values of 843.75 (SD ± 281.25) and 1734.375 (SD ± 46.875). But in the female PND7 group the polyI:C day 9 measurements show a higher bandwidth than the polyI:C day 15 group, with values of 2062.5 (SD ± 187.5) and 1218.75 (SD ± 93.75), respectively. These effects have decreased during the PND14 measurements.
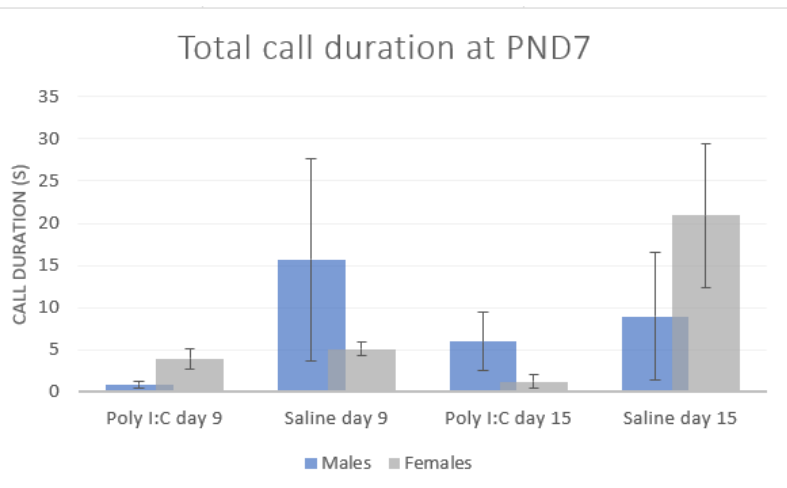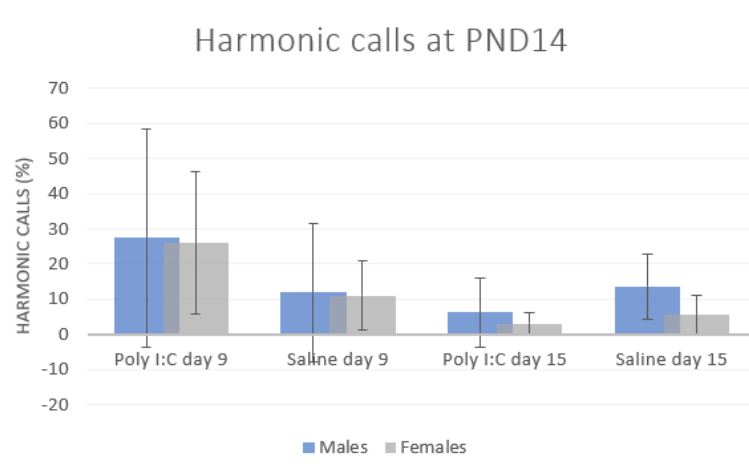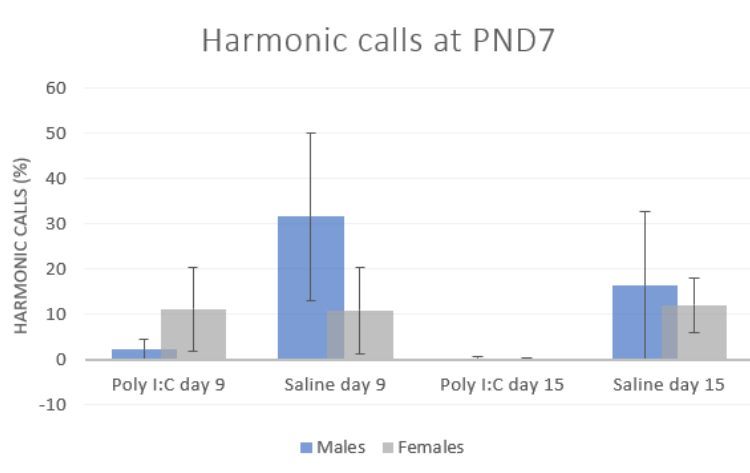

Figure 8a: The mean harmonic calls ± SD as percentage of the total calls at PND7 for the treatment groups polyI:C day 9, polyI:C day 15, saline day 9 and saline day 15. Males and females are separately shown.

Figure 8b: The mean harmonic calls ± SD as percentage of the total calls at PND14 for the treatment groups polyI:C day 9, polyI:C day 15, saline day 9 and saline day 15. Males and females are separately shown.

Figures 8a and 8b show the percentual number of harmonic calls at PND7 and PND14 for the four treatment groups. With p-values of 0,447 and 0,406 for sex and treatment*treatment day respectively, no significance could be found. However, when looking at the graphs, there seems to be a correlation between maternal polyI:C infections and the number of harmonic calls, especially for polyI:C at GD 15. This can especially be seen in the PND7 measurements, but the polyI:C day 15 group also shows less harmonic calls in the PND14 group. However, these values did not show significance either.

4   DISCUSSION

The present study explored the relationship between the moment of viral infections during pregnancy and the development of psychiatric disorders in offspring. For that, polyI:C was used to simulate viral infections in rats and the HRV and USV's were used as a measure for psychiatric disorders. We could only prove a significant difference between male and female rats at GD9 of injection in terms of the bandwidth, representing the complexity of USV's. None of the other variables gave significant results.

4.1 HEART RATE VARIABILITY

Even though significance was discarded during the post-hoc test, in the initial statistics it was observed that the RMSSD was slightly higher for females than for males when the mother of the rats was exposed to polyI:C during pregnancy. This was true for both polyI:C groups (GD9 and GD15) and for both measurements in the pup (PND7 and PND14). Even though the standard deviations are high, these results spark the idea that exposure to polyI:C during pregnancy increases the parasympathetic influence on the heart in females, but not in males. The HF also represents parasympathetic activity, but unfortunately no such correlation could be seen in the HF data. The LF and LF/HF data didn't show a clear correlation either.

These results are partially in line with previous research, because no difference has been found in LF/HF in people with anxiety disorders and some studies showed a decrease in HF in the presence of psychiatric disorders, when others didn't. (Schiweck et al., 2019; Cheng et al., 2022) But one study showed lower RMSSD and HF and higher LF in the presence of anxiety disorder symptoms in rats (Carnevali et al., 2014)

These things couldn't be proven in the current study and the exact correlation unfortunately couldn't be established due to high variance. Because there seems to be a difference amongst males and females, it is interesting to perform more research about this subject, especially because male-female differences in this topic haven't been extensively studied yet.

4.2 ULTRASONIC VOCALIZATIONS

The USV results also showed slight differences in the outcomes between the two sexes. The bandwidth was significantly different between males and females when injected at GD9. The results show that polyI:C at GD 9 gives females that produce a bigger bandwidth in their calls, and thus more complex calls. When polyI:C is injected at GD15, it gives males that produce more complex calls. However, this is only true for PND7. At PND14, no clear relationship could be seen. This is in line with previous studies that also saw a decrease in USV complexity early after birth after maternal polyI:C exposure, but no significant differences on PND14 anymore. (Haddad et al., 2020) But other studies contradict this idea, as they have found the bandwidth stays similar. (Potasiewicz et al., 2020) Yet another study described that males typically have lower bandwidths in their calls than females. (Lenell et al., 2021) That relationship was generally present in the saline groups in this study, however the saline PND7 day 15 group showed higher means for males than females. Because the variances for every treatment group are high, it remains difficult to draw conclusions from these findings.

None of the other USV results showed any significance, but when looking at graphs, a few things stand out. First of all, the total number of calls and the total call duration for both sexes seems to be lower on average for the polyI:C groups than for the saline groups at PND7. This corresponds to most other studies that have been done in this field, because mostly a decrease in USV's was seen after maternal polyI:C exposure during GD15. (Haddad et al., 2020; Jouda et al., 2019; Potasiewicz et al., 2020) For males, polyI:C at GD9 gave a substantial decrease in calls, while for females, polyI:C at GD15 gave a big decrease. At PND14, the number of calls in the saline group has decreased drastically compared to PND7, while they stay about the same for both polyI:C groups. Most other researchers haven't explicitly looked at sex differences. One study that did, could not find any sex differences in USV parameters, except for the total amount of calls. (Potasiewicz et al., 2020) Males would emit more calls, as was also described in another study (Lenell et al., 2021). This correlation couldn't been proven in the current research. Other studies have found no significant sex differences at all. (Stark et al., 2020)

No significance was found for the total call duration. The figures show something that looks like a correlation, but the proportions are very similar to the total number of calls. Therefore we presume that the differences are the result of the number of calls rather than the individual calls having a notable different length. These outcomes correspond with other studies. (Potasiewicz et al., 2020)

The harmonic call data shows that the polyI:C day 15 group at PND7 barely has harmonic calls. At PND14, some harmonics could be measured, but on average there are still less than in the other groups. In this study, we only looked at the percentual number of harmonic calls relative to the total amount of calls per pup. It is somewhat difficult to compare the results, because most previous studies didn't pay attention to harmonic syllables in pup USV's. There was one study in mice that described male pups to have less harmonic syllables with maternal polyI:C treatment. (Malkova, 2012) This was also observed in the present study, with both males and females. Even though we could not establish a significant effect, this indicates that such a correlation could be present.

What we also saw, was a highly varying pattern of harmonic calls in each pup. Some pups had none of these calls at all, whilst others had 4 or 5 harmonic calls in a row. There were also pups that made harmonic calls at random and there were four pups that had 50-65% of harmonic calls, 2 of them were in the polyI:C day 9 group and 2 were in the saline day 9 group.

Not only the harmonic syllables seemed to show patterns, differences in all vocalizations were observed. For instance, 'split calls' were seen, where a call seemingly stopped abrupt to start at the same moment at another frequency. There were more call structures that appeared a lot. Research has showed that USV's serve as communication tool, designed to give other rats information about things as food or predators, but it can also reflect an emotional state. (Takahashi et al., 2010) The call structures could therefore be correlated to psychiatric disorders too and perhaps they give additional information about such disorders, as has already been shown in older rats that have been exposed to maternal immune activation by polyI:C. Here, only the amount of 22 kHz calls were increased and not the 50 kHz calls, which means that it's correlated to negative emotions, such as fear. (Jouda et al., 2019) The current research didn't focus on that, but it would be interesting to elaborate on this in further research.

Multiple previous studies have shown that differences in USV's can be seen at PND7, but not at PND14. (Haddad et al., 2020; Wilkin-Krug et al., 2022) Even though pups haven't stopped completely with their isolation-induced USV's at that age, they have decreased with over 50% compared to PND7. That could be related to the rats being more independent from their mother at that age, especially with regard to the thermoregulation. That causes them to give less distress signals, which makes examination of the calls possible to a lesser extent. (Start et al., 2020) That means that PND7 would be a better day to measure USV's in.

4.3 CORRELATION HRV AND USV

Out of all the variables that were tested, only the USV's bandwidth showed significance. The USV data contained fewer measurements than we hoped for, but still gave an idea about possible sex differences that could be further established. In contrast, the HRV only showed a slight non-significant sex difference in the RMSSD data. This could mean that the HRV is just not suitable for measuring psychiatric diseases, but previous research does actually show significant correlations, so this speculation is unlikely. (Park et al., 2017; Liu et al., 2013; Carnevali et al., 2014) Another possibility is that perhaps the dose of polyI:C wasn't high enough to induce changes in the HRV. There are hardly any previous studies that look at the HRV in rats in the context of psychiatric disorders. Because most of the existing studies selected the animals on behavioral symptoms of these disorders, they didn't

use polyI:C injections. Therefore, it is not known what the ideal dose is for these kinds of measurements.

Even though the HRV results are somewhat disappointing in that sense, some ideas for sex differences could be found. The RMSSD showed a possible connection between females having a higher parasympathetic influence than males after polyI:C injection. This could indicate a larger HRV in females, which would mean that psychiatric disorders are less likely to develop. But this is highly speculative and it doesn't correspond with the USV data, that seems to suggest the idea of sex differences to be at least partly the result of the differences in the moment of injection.

When looking at only the saline results, the total number of calls and the total call duration seem to be higher in males in the day 9 group, while they both are higher in females in the day 15 group, especially in the PND7 results. Also, females show a bigger bandwidth in the saline day 9 group, but there are no clear differences in the saline day 15 groups. These differences can also be seen in the RMSSD PND14 measurements, as well as in the HF PND14 and LF and LF/HF at PND7 and PND14 measurements. Note that these differences are not significant, but that they could mean that the stress from the injection has influenced the results too. These two saline groups now show different results and that could interfere with the results from the polyI:C groups, so it would have been good to have a baseline group too, without any injections. Previous research showed that maternal stress during pregnancy reduces the amount of USV's in the pups. They couldn't find any sex differences, but the stress wasn't tested on different days of pregnancy, but rather during the entire pregnancy. (Morgan & Frye, 1999) Perhaps the stress from the injection at a certain time point during pregnancy can also induce differences between males and females. (Cui et al., 2009)

4.4 LIMITATIONS

The experiments were not without limitations. First of all, the measurements were done in a somewhat stressful environment for the pups. We attempted to reduce the amount of stress as much as possible by heating up the base plates of the ECGenie, handling the pups with care and ensuring that the pups were separated from the rest of the litter for just 10 to 15 minutes. Nevertheless, stress has undeniably occurred and this can influence the amount and patterns of ultrasonic vocalizations. (Pertsov et al., 2012) It would have been best to measure a baseline too, but in the current setup it was not possible to do this without having the rest of the litter interfere with the signal.

It is also known that the heart rate differs when the animal is experiencing stress. (Vetulani, 2013) Furthermore, the heart rate variability varies over different days, moments of the day and over lifetime. (Hayano & Yuda, 2022) To reduce these external influences as much as possible, all pups were measured on the same postnatal days, so they would be equal in development. Also, all measurements were done in the morning, preferably between 10:00 and 11:00. But we did only measure each rat for 8 minutes at two different time points. With 16 minutes of total data per rat, we will never be able to see the full influence that maternal polyI:C injections could have on the pups. In this setup it is not possible to prolong the measurement without maternal separation becoming an important factor. But in further research, it would be good to analyse the 24-hour HRV.

Even though most of the results were not significant, correlations seemed to be present. In the current experiments we ended up with less rats and less measurements than originally planned. It is likely that this gave a big increase in the variance and thereby in the standard deviations.

We were aiming for an amount of 20 litters in total, which would give 10 infant males and 10 infant females per group. Unfortunately, we ended up with 15 litters in total because of a few unrelated complications during pregnancy or birth. On the first day and during almost half of the measurements

of cohort 2, the microphone didn't record the calls properly. This was probably due to a problem with the hardware. Therefore, the PND7 measurements were largely unusable, leaving only 4 USV measurements for both the polyI:C day 9 and polyI:C day 15 groups. That means we only had 2 males and 2 females in those groups that also came from the same litter. Even though all rats are bred at site and should be genetically identical to each other, the amount of grooming by the mother can also influence the amount of USV's from the pups. (Brunelli et al., 2015) Those PND7 groups can still be used to generate an idea, but the sample size is so small that no definite conclusion can be drawn from it. The PND14 USV data is more usable, because those polyI:C groups still have 16 measurements, which is more reliable.

For the HRV data, 3 measurements were excluded for a lack of reliable intervals and 3 more were excluded after analysis. They showed large RMSSD numbers in all intervals. After a second look, these pups all appeared to have an arrhythmia. It was interesting to see that the PND7 measurements of the same pups did have usable data, with normal RMSSD values. That indicates that the arrhythmia must have developed between PND7 and 14, or that it is a paroxysmal arrhythmia, that doesn't always show. It is known that after birth, a rat's heart becomes stronger with myocyte hypertrophy and the heart rate goes up. (Vornanen, 1996) Also, β-adrenergic receptors, synaptic vesicles, sympathetic fibres and ganglia keep developing after birth. In addition, the parasympathetic system is not completely developed yet in newborn rats, which could influence the heart rate, and the heart rate variability. (Quigley & Myers, 1996) The sarcoplasmic reticulum will only be fully developed after two or three weeks after birth. This influences the calcium regulations in the heart and therefore the heart rate. That could explain why malfunctions in the heart rate are only showing at PND14. (Vornanen, 1996) As the heart is not completely matured yet at PND7, PND14 would be a better day to measure the HRV in.

Even though we used less animals, the groups with the most animals still showed a lot of variance. That indicates that the used parameters are highly variant over individuals as well as groups, which means a larger sample size would be preferred in further experiments.

The software that was used for the USV data analysis was Raven Pro. This program is not able to automatically analyse the measurements, which means it had to be done manually. The effects of human error were decreased as much as possible by selecting the data on the specified parameters afterwards and thereby excluding calls that don't comply with these parameters. But an additional limitation was the noise that was generated by the microphone, which was present in all measurements between 30 and 55 kHz. This complicated the analysis of the file and some calls in that range were possibly missed.

There are more factors that could influence the occurrence of psychiatric disorders in offspring, like preterm birth and complications during pregnancy, but also rat breed, age and living circumstances. (Khandaker et al., 2013; Jouda et al., 2019) The rats used in this experiment, should all be genetically similar, but other things can influence the results too. It has been shown that maternal infections with polyI:C can give a reduced expression of the P-gp protein in the human placenta. As P-gp functions as an efflux pump in both the blood brain barrier (BBB) and the placenta barrier, a reduced expression can lead to a decrease in foetal protection against xenobiotics and environmental toxins. This may have teratogenic consequences, which might also lead to changes in the brain, promoting mental illnesses. (Bloise et al., 2017) It is unknown how rat placenta's are influenced and there shouldn't have been any toxins or xenobiotics present in the rats' surroundings, but it is good to keep these variables in mind, especially when interpolating the data to the human situation.

It should also be noted that a rodent placenta differs from human placenta's in endocrine and immunological functions too. Also, there is a difference in the crossing of molecules over the placenta.

This means that the outcomes can't fully be extrapolated to humans. However, both human and rat studies have demonstrated a relationship between maternal infections and mental illnesses in offspring, indicating that there is enough overlap to use rodents as a model for the human situation. (Meyer et al., 2009)

Lastly, the foetal response to maternal immune activation at specific moments can't easily be imitated in rodent experiments. This is difficult because rodents have a delayed immune development compared to humans. (Haddad et al., 2020) We tried to correct for that as much as possible by rearranging the trimesters of pregnancy, but there will still be differences. Therefore the rodent model can only function to give an idea about the human outcomes.

In summary, the use of rats instead of humans presents advantages and disadvantages. To give the best idea of the actual effects, these two studies should be combined. In that way, a correction can be made for both the variables in human studies and for the differences between rats and humans.

4.5 FUTURE STUDIES

For future studies, the uncertainties that still remain now can be further analysed. As a substantial amount of variance has been seen in both the USV and HRV data, it is key to use more animals than in the current study. With a decreased variance, important correlations could come to light, not only regarding differences in time of injection, but also in sex differences. Especially the HRV is a relatively new parameter to study this topic in and more studies can certainly give more insights. As there is a discussion about the HRV being suitable as a diagnostic parameter for psychiatric disorders in humans, rodent experiments are very helpful to clarify uncertainties. (Dormal et al., 2021)

It would also be interesting to look at USV's and the HRV in infant rodents in correlation to behavioural changes later in life. Maternal immune activation could increase the presence on psychiatric diseases, but that doesn't mean that all pups develop them. Most likely, a larger amount of the pups will develop these disorders, but there will be a part that stays healthy, despite maternal immune activation. These healthy pups can decrease the total observed effect, which could explain the high variances that studies in this field typically show. By comparing infant USV's and HRV of offspring with psychiatric characteristics later in life with offspring that behaves in a healthy manner, more insights could be gained. In this way, the correlation between late onset of schizophrenia and early HRV and USV changes could also be observed. (Albert & Weibell, 2019)

Another question that still remains is if there are any differences in HRV between the multiple psychiatric disorders that exist, or perhaps between groups of psychiatric disorders.
Previously, a reduced HRV has been correlated to depression, schizophrenia and ASD. However, ASD showed less reduction. (Schiweck et al., 2019; Haigh et al., 2021)
A meta-analysis amongst humans showed the differences in HRV in people with ASD, compared to people without this disorder. They found that social stress gives a significant reduction of the HRV in people with ASD, but the baseline HRV is also significantly lower. This is probably correlated with an hyperactivity of the prefrontal cortex, the insula and the anterior cingulate cortex in the brain. (Cheng et al., 2020) It is difficult and maybe even impossible to differentiate ASD from other psychiatric disorders using only HRV, as the HRV is lowered in other psychiatric disorders as well (Schiweck et al., 2019; Haigh et al., 2021)
However, the fact that only social tasks and not cognitive tasks showed a decrease in the HRV can give insights too. It is thought that the vagal branch from the nucleus ambiguous, which is associated with affiliative behaviour, does not work properly in people with ASD. This could be associated with early

developmental issues in the brain, especially the myelinated vagal system and the cortical regulation of the brainstem areas. So by measuring the HRV in specific situations, different psychiatric disorders might still be distinguishable from one another in the future. (Cheng et al., 2020)

After the effects of maternal immune stimulation as result of viral infections are clear, research should focus on the prevention of psychiatric disorders in offspring. Promising results have arisen from the use of probiotics, antibodies, specific cytokines, environmental enrichment, dietary supplementation and antipurinergic therapy in offspring, to decrease or even reverse the development of several psychiatric disorders. (Estes & Mcallister, 2016) More research should be conducted to gain knowledge about the applicability, the efficacy and individuals that are most at risk. It would be good to be able to map children that are at risk in the future, after which they can be further examined with methods that don't fully depend on questionnaires.

5 CONCLUSION

This study assessed the relationship between immune activation in pregnant rats and the influence on the development of psychiatric disorders in offspring. Specifically, we looked at the differences between immune activation between the first and second trimester of pregnancy and the impact that it had on ultrasonic vocalizations and the heart rate variability in offspring.

We found a significant interaction between the sex of the pup and the treatment that the mother had, but only in terms of bandwidth, representing the complexity of ultrasonic vocalizations. Even though we did not find any other significant correlations, the means of every group did show some more differences between the sexes. The USV data seemed to suggest that males are more affected by maternal polyI:C exposure on GD9, while females seemed to be more affected at exposure on GD15. That was true for the total number of calls, the total duration of calls and for the harmonic calls. This developed the idea that the moment of polyI:C injection determines if males or females will be more affected. However, the HRV data didn't show that correlation. Most variables didn't have any noticeable differences, but for the RMSSD a small sex difference could be observed: females had a higher RMSSD, and thus a higher parasympathetic influence, in the polyI:C groups, whereas males had higher RMSSD values in the control groups.
If the sample size would have been bigger, we presume we could have been able to find more significance. Additional research is important to further establish these relationships. In that way we hope to eventually be able map children at risk of psychiatric disorders, to start treatment early and hence, decrease the severity of these diseases.

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7 ATTACHMENTS

7.1 APPENDIX A

Table 1: The temperature of the seven rats in the first cohort in degrees Celsius, measured immediately before (0) the injection and 2, 4 and 24 hours after the injection. The rat identification numbers and injection details are included.

Treatment	Rat	Time (hours)			
		0	2	4	24
PolyI:C day 9	26047	28.0	28.8	28.8	28.6
PolyI:C day 9	25995	27.5	28.3	29.3	27.8
Saline day 9	26065	27.0	27.6	27.3	27.6
PolyI:C day 15	26005	29.3	29.5	29.0	28.5
Saline day 15	26006	28.9	28.4	28.8	29.4
Saline day 15	26004	29.2	29.1	29.1	28.6

Table 2: The temperature of the eight rats in the second cohort in degrees Celsius, measured immediately before (0) the injection and 4 and 24 hours after the injection. The rat identification numbers and injection details are included.

Treatment	Rat	Time (hours)		
		0	4	24
PolyI:C day 9	26007	27,6	29,7	28,8
PolyI:C day 9	25993	29,0	29,9	29,9
Saline day 9	15717	30,4	30,4	30,3
Saline day 9	15936	30,5	30,2	30,7
Saline day 9	26092	26,8	26,5	27,2
PolyI:C day 15	26093	27,3	29,3	27,1
PolyI:C day 15	26094	26,8	31,1	27,9
PolyI:C day 15	15939	26,5	28,7	27,0

Table 3: The weight of the seven rats in the first cohort in milligrams, measured immediately before (0) the injection and 24 hours after injection. The rat identification numbers and injection details are included.

Treatment	Rat	Time (hours)	
		0	24
PolyI:C day 9	26047	305	314
PolyI:C day 9	25995	295	292
Saline day 9	26065	305	303
PolyI:C day 15	26005	323	321
PolyI:C day 15	26064	294	283
Saline day 15	26006	321	325
Saline day 15	26004	306	312

* Unfortunately, the weight data from the second cohort got lost during the experiment.

7.2 APPENDIX B

Table 4: overview over the timeline of the first cohort, including the treatment that each animal received. The dates of pregnancy, injection, birth and the two analyses are mentioned. The rat that had to be excluded in the experiment is also displayed in this table.

Rat	Date pregnancy	Date injection	Type injection	Pups born	Analysis 1	Analysis 2
26047	5 oct 2022	14 oct 2022	PolyI:C 2 mg/kg (day 9)	27 oct 2022	2 nov 2022	9 nov 2022
25995	5 oct 2022	14 oct 2022	PolyI:C 2 mg/kg (day 9)	28 oct 2022	3 nov 2022	10 nov 2022
26065	5 oct 2022	14 oct 2022	Saline 0,9% (day 9)	28 oct 2022	3 nov 2022	10 nov 2022
26064	6 oct 2022	21 oct 2022	polyI:C 2 mg/kg (day 15)	-*	-	-
26005	6 oct 2022	21 oct 2022	PolyI:C 2 mg/kg (day 15)	29 oct 2022	4 nov 2022	11 nov 2022
26004	6 oct 2022	21 oct 2022	Saline 0,9% (day 15)	29 oct 2022	4 nov 2022	11 nov 2022
26006	7 oct 2022	22 oct 2022	Saline 0,9% (day 15)	29 oct 2022	4 nov 2022	11 nov 2022

* Rat 26064 wasn't pregnant after all.

Table 5: overview over the timeline of the second cohort, including the treatment that each animal received. The dates of pregnancy, injection, birth and the two analyses are mentioned. The rats that had to be excluded in the experiment are also displayed in this table.

Rat	Date pregnancy	Date injection	Type injection	Pups born	Analysis 1	Analysis 2
26007	28 oct 2022	06 nov 2022	PolyI:C 2 mg/kg (day 9)	19 nov 2022	25 nov 2022	02 dec 2022
25993	28 oct 2022	06 nov 2022	PolyI:C 2 mg/kg (day 9)	20 nov 2022	26 nov 2022	03 dec 2022
25718	28 oct 2022	06 nov 2022	PolyI:C 2 mg/kg (day 9)	21 nov 2022	-*	-
26093	29 oct 2022	13 nov 2022	PolyI:C 2 mg/kg (day 15)	19 nov 2022	25 nov 2022	02 dec 2022
26094	29 oct 2022	13 nov 2022	PolyI:C 2 mg/kg (day 15)	18 nov 2022	24 nov 2022	01 dec 2022
25937	29 oct 2022	13 nov 2022	PolyI:C 2 mg/kg (day 15)	-**	-	-
25939	29 oct 2022	13 nov 2022	PolyI:C 2 mg/kg (day 15)	20 nov 2022	26 nov 2022	03 dec 2022
26616	29 oct 2022	13 nov 2022	Saline 0,9% (day 15)	-***	-	-
25717	30 oct 2022	08 nov 2022	Saline 0,9% (day 9)	24 nov 2022	30 nov 2022	07 dec 2022
25938	31 oct 2022	15 nov 2022	Saline 0,9% (day 15)	24 nov 2022****	-	-
25936	01 nov 2022	10 nov 2022	Saline 0,9% (day 9)	23 nov 2022	29 nov 2022	06 dec 2022
26092	04 nov 2022	13 nov 2022	Saline 0,9% (day 9)	26 nov 2022	02 dec 2022	09 dec 2022
25994	-*****	-	-	23 nov 2022	29 nov 2022	06 dec 2022

* Rat 25718 only had 1 dead pup left after a few days.
** Rat 25937 wasn't pregnant after all.
*** Rat 26616 had to be euthanized halfway through the experiment.
**** Rat 25938 only had 1 pup left at postnatal day 7. It won't be analyzed.
***** Rat 25994 didn't have a plug, but got pregnant. The pups will be analyzed.

7.3 APPENDIX C

Table 6: Overview over the mean HRV data that is used for generating the graphs in the 'result' section. The mean RMSSD, HF power, LF power and LF/HF are displayed for every treatment group.

	Mean RMSSD	Mean HF power (%)	Mean LF power (%)	Mean LF/HF
PolyI:C day 9 PND7 males	1,069	40,217	5,149	0,169
PolyI:C day 9 PND7 females	1,166	41,195	6,165	0,159
Saline day 9 PND7 males	0,979	52,087	6,543	0,147
Saline day 9 PND7 females	0,925	45,875	7,436	0,189
PolyI:C day 15 PND7 males	1,037	50,127	5,760	0,117
PolyI:C day 15 PND7 females	1,050	51,407	6,464	0,145
Saline day 15 PND7 males	1,139	48,669	6,894	0,140
Saline day 15 PND7 females	1,003	49,329	3,468	0,089
PolyI:C day 9 PND14 males	1,149	38,422	10,495	0,361
PolyI:C day 9 PND14 females	1,257	43,665	12,493	0,336
Saline day 9 PND14 males	1,126	44,429	10,843	0,289
Saline day 9 PND14 females	1,097	39,783	19,378	0,569
PolyI:C day 15 PND14 males	1,078	43,459	12,043	0,382
PolyI:C day 15 PND14 females	1,238	32,802	16,984	0,543
Saline day 15 PND14 males	1,587	25,963	14,310	0,880
Saline day 15 PND14 females	1,068	48,031	12,036	0,396

Table 7: Overview over the mean USV data that is used for generating the graphs from the 'result' section. The mean total number of calls, total call duration, bandwidth 50% and percentual harmonic calls are displayed for treatment group.

Group	Mean number of calls	mean call duration (s)	Mean bandwidth 50% (Hz)	Mean percentual harmonizations (%)
PolyI:C day 9 PND7 males	27,0	0,852	843,750	2,174
PolyI:C day 9 PND7 females	86,5	3,908	2062,500	11,074
Saline day 9 PND7 males	293,5	15,674	726,563	31,547
Saline day 9 PND7 females	107,8	5,104	1453,125	10,878
PolyI:C day 15 PND7 males	117,5	5,966	1734,375	0,263
PolyI:C day 15 PND7 females	25,5	1,234	1218,750	0,131
Saline day 15 PND7 males	176,0	8,968	1125,000	16,272
Saline day 15 PND7 females	416,8	20,909	984,375	11,992
PolyI:C day 9 PND14 males	59,4	6,206	1296,875	27,371
PolyI:C day 9 PND14 females	80,6	8,458	1625,000	25,965
Saline day 9 PND14 males	61,0	3,210	1171,875	11,842
Saline day 9 PND14 females	44,4	1,948	1839,844	11,064
PolyI:C day 15 PND14 males	96,8	4,828	1570,313	6,198
PolyI:C day 15 PND14 females	37,4	1,628	1558,594	3,101
Saline day 15 PND14 males	18,8	0,692	1500,000	13,566
Saline day 15 PND14 females	32,0	1,675	1593,750	5,439

7.4 APPENDIX D

Table 8: The fixed effect Omnibus test as result of the mixed model linear regression of the logarithm of the RMSSD values. The F-value, the degrees of freedom and the p-value are stated for every variable, including every interaction for these variables.

Fixed Effect Omnibus tests

	F	Num df	Den df	p
Sex	0.985	1	90.0	0.324
PND	6.780	1	90.0	0.011
Treatment	0.423	1	90.0	0.517
Treatment day	0.842	1	90.0	0.361
Sex ⁂ PND	0.395	1	90.0	0.531
Sex ⁂ Treatment	6.637	1	90.0	0.012
PND ⁂ Treatment	0.612	1	90.0	0.436
Sex ⁂ Treatment day	0.625	1	90.0	0.431
PND ⁂ Treatment day	0.136	1	90.0	0.713
Treatment ⁂ Treatment day	4.560	1	90.0	0.035
Sex ⁂ PND ⁂ Treatment	1.437	1	90.0	0.234
Sex ⁂ PND ⁂ Treatment day	5.73e−4	1	90.0	0.981
Sex ⁂ Treatment ⁂ Treatment day	0.330	1	90.0	0.567
PND ⁂ Treatment ⁂ Treatment day	0.103	1	90.0	0.749
Sex ⁂ PND ⁂ Treatment ⁂ Treatment day	0.323	1	90.0	0.572

Note. Satterthwaite method for degrees of freedom

Table 9: Bonferroni post-hoc test for interaction sex * treatment, with all individual interactions between the study groups and corresponding p-values.

Post Hoc Comparisons - Sex ⁂ Treatment

Comparison								
Sex	Treatment		Sex	Treatment	Difference	SE	t	df	$p_{bonferroni}$
Female	PolyIC	-	Female	Saline	0.06515	0.0285	2.285	30.0	0.178
Female	PolyIC	-	Male	Saline	−0.00691	0.0279	−0.248	28.3	1.000
Male	PolyIC	-	Female	PolyIC	−0.03197	0.0263	−1.214	39.2	1.000
Male	PolyIC	-	Female	Saline	0.03318	0.0294	1.128	31.9	1.000
Male	PolyIC	-	Male	Saline	−0.03888	0.0288	−1.350	30.3	1.000
Male	Saline	-	Female	Saline	0.07205	0.0308	2.337	36.5	0.150

Table 10: Bonferroni post-hoc test for interaction treatment * treatment day, with all individual interactions between the study groups and corresponding p-values.

Post Hoc Comparisons - Treatment ⁂ Treatment day

Comparison								
Treatment	Treatment day		Treatment	Treatment day	Difference	SE	t	df	$p_{bonferroni}$
PolyIC	15	-	Saline	15	−0.02998	0.0308	−0.972	9.12	1.000
PolyIC	9	-	PolyIC	15	0.02459	0.0263	0.934	10.43	1.000
PolyIC	9	-	Saline	15	−0.00539	0.0313	−0.172	9.45	1.000
PolyIC	9	-	Saline	9	0.05625	0.0262	2.144	10.28	0.341
Saline	9	-	PolyIC	15	−0.03166	0.0257	−1.232	9.78	1.000
Saline	9	-	Saline	15	−0.06163	0.0308	−2.004	9.03	0.456

Table 11: The fixed effect Omnibus test as result of the mixed model linear regression of the HF (%) values. The F-value, the degrees of freedom and the p-value are stated for every variable, including every interaction for these variables.

Fixed Effect Omnibus tests

	F	Num df	Den df	p
Sex	0.24020	1	46.2	0.626
PND	14.04750	1	40.4	< .001
Treatment	0.21137	1	43.6	0.648
Treatment day	0.00487	1	43.6	0.945
Sex ✻ PND	1.01609	1	40.7	0.319
Sex ✻ Treatment	0.60372	1	46.2	0.441
PND ✻ Treatment	0.75991	1	40.4	0.389
Sex ✻ Treatment day	0.32597	1	46.2	0.571
PND ✻ Treatment day	5.11199	1	40.4	0.029
Treatment ✻ Treatment day	0.60433	1	43.6	0.441
Sex ✻ PND ✻ Treatment	3.64423	1	40.7	0.063
Sex ✻ PND ✻ Treatment day	0.09713	1	40.7	0.757
Sex ✻ Treatment ✻ Treatment day	2.50969	1	46.2	0.120
PND ✻ Treatment ✻ Treatment day	1.23910	1	40.4	0.272
Sex ✻ PND ✻ Treatment ✻ Treatment day	3.60864	1	40.7	0.065

Note. Satterthwaite method for degrees of freedom

Table 12: The fixed effect Omnibus test as result of the mixed model linear regression of the LF (%) values. The F-value, the degrees of freedom and the p-value are stated for every variable, including every interaction for these variables.

Fixed Effect Omnibus tests

	F	Num df	Den df	p
Sex	0.62160	1	81.72	0.433
PND	37.88217	1	81.50	< .001
Treatment	0.00714	1	9.99	0.934
Treatment day	0.11183	1	9.99	0.745
Sex ✻ PND	1.08684	1	81.59	0.300
Sex ✻ Treatment	2.25172	1	81.72	0.137
PND ✻ Treatment	0.22007	1	81.50	0.640
Sex ✻ Treatment day	2.28195	1	81.72	0.135
PND ✻ Treatment day	4.38e−5	1	81.50	0.995
Treatment ✻ Treatment day	3.68165	1	9.99	0.084
Sex ✻ PND ✻ Treatment	0.61775	1	81.59	0.434
Sex ✻ PND ✻ Treatment day	1.05188	1	81.59	0.308
Sex ✻ Treatment ✻ Treatment day	1.73048	1	81.72	0.192
PND ✻ Treatment ✻ Treatment day	0.00259	1	81.50	0.960
Sex ✻ PND ✻ Treatment ✻ Treatment day	0.01083	1	81.59	0.917

Note. Satterthwaite method for degrees of freedom

Table 13: The fixed effect Omnibus test as result of the mixed model linear regression of the LF/HF values. The F-value, the degrees of freedom and the p-value are stated for every variable, including every interaction for these variables.

Fixed Effect Omnibus tests

	F	Num df	Den df	p
Sex	0.14600	1	43.9	0.704
PND	36.23997	1	41.7	< .001
Treatment	0.04448	1	42.6	0.834
Treatment day	0.00226	1	42.6	0.962
Sex ✴ PND	0.15066	1	42.4	0.700
Sex ✴ Treatment	1.21549	1	43.9	0.276
PND ✴ Treatment	0.39104	1	41.7	0.535
Sex ✴ Treatment day	1.22578	1	43.9	0.274
PND ✴ Treatment day	0.62888	1	41.7	0.432
Treatment ✴ Treatment day	0.75502	1	42.6	0.390
Sex ✴ PND ✴ Treatment	0.01031	1	42.4	0.920
Sex ✴ PND ✴ Treatment day	0.18692	1	42.4	0.668
Sex ✴ Treatment ✴ Treatment day	1.59163	1	43.9	0.214
PND ✴ Treatment ✴ Treatment day	0.01064	1	41.7	0.918
Sex ✴ PND ✴ Treatment ✴ Treatment day	0.64116	1	42.4	0.428

Note. Satterthwaite method for degrees of freedom

7.5 APPENDIX E

Table 14: The fixed effect Omnibus test as result of the generalized mixed model linear regression, specifically the negative binominal, of the total number of calls. The F-value, the degrees of freedom and the p-value are stated for every variable, including every interaction for these variables.

Fixed Effect Omnibus tests

	X²	df	p
Sex	0.08297	1.00	0.773
PND	52.23140	1.00	< .001
Treatment	0.21107	1.00	0.646
Treatment day	0.23837	1.00	0.625
Sex ✳ PND	0.00373	1.00	0.951
Sex ✳ Treatment	0.37131	1.00	0.542
PND ✳ Treatment	3.74188	1.00	0.053
Sex ✳ Treatment day	0.87914	1.00	0.348
PND ✳ Treatment day	1.97282	1.00	0.160
Treatment ✳ Treatment day	0.02901	1.00	0.865
Sex ✳ PND ✳ Treatment	0.02051	1.00	0.886
Sex ✳ PND ✳ Treatment day	0.00126	1.00	0.972
Sex ✳ Treatment ✳ Treatment day	22.78738	1.00	< .001
PND ✳ Treatment ✳ Treatment day	9.92056	1.00	0.002
Sex ✳ PND ✳ Treatment ✳ Treatment day	2.50244	1.00	0.114

Table 15: The fixed effect Omnibus test as result of the mixed model linear regression of the total call duration values. The F-value, the degrees of freedom and the p-value are stated for every variable, including every interaction for these variables.

Fixed Effect Omnibus tests

	F	Num df	Den df	p
Sex	0.02960	1	52.8	0.864
PND	26.79639	1	57.4	< .001
Treatment	0.45189	1	10.4	0.516
Treatment day	0.12207	1	10.4	0.734
Sex ✳ PND	0.00131	1	52.8	0.971
Sex ✳ Treatment	0.49805	1	52.8	0.483
PND ✳ Treatment	14.72052	1	57.4	< .001
Sex ✳ Treatment day	1.48243	1	52.8	0.229
PND ✳ Treatment day	0.02468	1	57.4	0.876
Treatment ✳ Treatment day	0.16829	1	10.4	0.690
Sex ✳ PND ✳ Treatment	0.07078	1	52.8	0.791
Sex ✳ PND ✳ Treatment day	0.10780	1	52.8	0.744
Sex ✳ Treatment ✳ Treatment day	20.06725	1	52.8	< .001
PND ✳ Treatment ✳ Treatment day	0.62764	1	57.4	0.431
Sex ✳ PND ✳ Treatment ✳ Treatment day	0.95471	1	52.8	0.333

Note. Satterthwaite method for degrees of freedom

Table 16: The fixed effect Omnibus test as result of the mixed model linear regression of the bandwidth 50% values. The F-value, the degrees of freedom and the p-value are stated for every variable, including every interaction for these variables.

Fixed Effect Omnibus tests

	F	Num df	Den df	p
Sex	3.60490	1	37.59	0.065
PND	8.73514	1	24.36	0.007
Treatment	0.19208	1	8.38	0.672
Treatment day	0.00425	1	8.38	0.950
Sex ✳ PND	2.02e−4	1	31.54	0.989
Sex ✳ Treatment	0.15343	1	37.59	0.697
PND ✳ Treatment	0.55722	1	24.36	0.463
Sex ✳ Treatment day	7.92833	1	37.59	0.008
PND ✳ Treatment day	0.03098	1	24.36	0.862
Treatment ✳ Treatment day	0.28316	1	8.38	0.608
Sex ✳ PND ✳ Treatment	0.42002	1	31.54	0.522
Sex ✳ PND ✳ Treatment day	1.69162	1	31.54	0.203
Sex ✳ Treatment ✳ Treatment day	0.00348	1	37.59	0.953
PND ✳ Treatment ✳ Treatment day	0.10022	1	24.36	0.754
Sex ✳ PND ✳ Treatment ✳ Treatment day	1.58366	1	31.54	0.217

Note. Satterthwaite method for degrees of freedom

Table 17: The fixed effect Omnibus test as result of the mixed model linear regression of the arcsine transformation for the harmonic call values. The F-value, the degrees of freedom and the p-value are stated for every variable, including every interaction for these variables.

Fixed Effect Omnibus tests

	F	Num df	Den df	p
Sex	0.59234	1	32.7	0.447
PND	1.56841	1	11.1	0.236
Treatment	1.14063	1	10.1	0.310
Treatment day	2.90896	1	10.1	0.119
Sex ✳ PND	0.22973	1	13.7	0.639
Sex ✳ Treatment	0.82220	1	32.7	0.371
PND ✳ Treatment	2.14841	1	11.1	0.171
Sex ✳ Treatment day	0.09022	1	32.7	0.766
PND ✳ Treatment day	0.95208	1	11.1	0.350
Treatment ✳ Treatment day	0.75269	1	10.1	0.406
Sex ✳ PND ✳ Treatment	0.95959	1	13.7	0.344
Sex ✳ PND ✳ Treatment day	0.62036	1	13.7	0.444
Sex ✳ Treatment ✳ Treatment day	0.63654	1	32.7	0.431
PND ✳ Treatment ✳ Treatment day	0.00172	1	11.1	0.968
Sex ✳ PND ✳ Treatment ✳ Treatment day	2.34144	1	13.7	0.149

Note. Satterthwaite method for degrees of freedom