The American Naturalist

# Social plasticity enhances signal-preference co-divergence

--Manuscript Draft--

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| **Manuscript Number:** | 61361R4 |
| **Full Title:** | Social plasticity enhances signal-preference co-divergence |
| **Short Title:** | Social plasticity enhances co-divergence |
| **Article Type:** | Major Article |
| **Additional Information:** | |
| **Question** | **Response** |
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Dear Prof. Lau,

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Jennifer Lau
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Sincerely
Bob Montgomerie
mont@queensu.ca

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# Social plasticity enhances signal-preference co-divergence

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**Keywords**: *indirect genetic effects, mating preference, courtship signal, Membracidae, vibrational communication*

**Word count:** 7,466

**Article type:** Major article

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The authors wish to be identified to the reviewers.

## Abstract

The social environment is often the most dynamic and fitness-relevant environment animals experience. Here we tested whether plasticity arising from variation in social environments can promote signal-preference divergence—a key prediction of recent speciation theory, but one that has proven difficult to test in natural systems. Interactions in mixed social aggregations could reduce, create or enhance signal-preference differences. In the latter case, social plasticity could establish or increase assortative mating. We tested this by rearing two recently diverged species of *Enchenopa* treehoppers—sap-feeding insects that communicate with plant-borne vibrational signals—in treatments consisting of mixed-species versus own-species aggregations. Social experience with heterospecifics (in the mixed-species treatment) resulted in enhanced signal-preference species differences. For one of the two species, we tested but found no evidence for differences in the plastic response between sympatric and allopatric sites suggesting the absence of reinforcement in the signals and preferences and their plastic response. Our results support the hypothesis that social plasticity can create or enhance signal-preference differences, and that this might occur in the absence of long-term selection on plastic responses themselves. Such social plasticity may facilitate rapid bursts of diversification.

**Secondary abstract in Spanish:**

## La plasticidad social aumenta la co-divergencia entre señales y preferencias

El ambiente social es a menudo dinámico e influye la adecuación de los animales que contiene. En agregaciones mixtas, por ejemplo, las interacciones entre individuos podrían influir las diferencias entre señales de cortejo y preferencias de pareja— creándolas, aumentándolas, o reduciéndolas—y afectar los patrones de aislamiento reproductivo entre poblaciones naturales. Aquí pusimos a prueba esta hipótesis. Condujimos un experimento con dos especies recientemente divergidas de membrácidos del complejo Enchenopa binotata—insectos que se alimentan de la savia de sus plantas hospederas y que se comunican con señales vibracionales que se transmiten por los tejidos de las plants. Creamos tratamientos de agregaciones mixtas o de cada una de las especie, en los cuales los membrácidos se desarrollaron. Encontramos mayores diferencias en señales y preferencias entre las dos especies en el tratamiento mixto. En una de las especies, no hubo diferencia en la respuesta plástica a los tratamientos entre individuos de sitios simpátricos y alopátricos, lo cual sugiere tentativamente que la respuesta plástica que observamos no se debe a selección previa para prevenir la hibridización. (No logramos hacer esta prueba para la otra especie.) Estos resultados apoyan a la hipótesis que dice que la plasticidad debida al ambiente social puede crear o aumentar diferencias en señales y preferencias al ocurrir primeros encuentros entre poblaciones en divergencia o especies recientemente formadas. Tal plasticidad podría resultar en episodios de diversificación rápida.

**Secondary abstract in French:**

## La plasticité sociale augmente la co-divergence des signaux et des préférences

L'environnement social est souvent l'environnement le plus dynamique et le plus pertinent pour la valeur sélective que les animaux éprouvent. Dans cet article, nous avons testé si la plasticité due à la variation de l'environnement social peut promouvoir la divergence des signaux et des préférences—une prédiction clé de la théorie de spéciation récente mais qui s'est révélée difficile à tester en milieux naturel. Les interactions en aggrégation sociales mixtes pourraient réduire, créer ou augmenter les différences de signaux et de préférences. Dans le dernier cas, la plasticité sociale pourrait établir ou augmenter l'accouplement assortatif. Nous avons testé cela en élevant deux espèces récemment divergées de Membracides du genre *Enchenopa*—des insectes se nourrisant de sève qui communique grâce à des signaux vibratoires transmis dans les plantes—dans des traitements expérimentaux consistant d'aggregations d'un mixe des deux espèces et d'une seule espèce. L'expérience sociale d'hétérospécifiques (dans le traitement mixe) a augmenté les différences de signaux et préférences entre espèces. Pour l'une des espèces, nous avons testé la présence de différences de réponse plastique entre les sites en allopatrie et en sympatrie mais nous n'en avons pas trouvé suggérant l'absence de renforcement des signaux et préférences et leur réponse plastique. Nos résultats soutiennent l'hypothèse d'une plasticité qui peut créer ou augmenter les différences de signaux et préférences et le fait que cela peut se produire en l'absence de sélection à long terme sur les réponses plastiques. Une telle plasticité sociale pourrait faciliter une diversification "en rafales".

## Introduction

Phenotypic plasticity in sexual traits such as advertisement signals and mate preferences may have important consequences for speciation. Speciation involves the establishment of linkage disequilibrium in polygenic suites of ecological and sexual traits (Kirkpatrick and Ravigné 2002; Coyne and Orr 2004; van Doorn et al. 2009; Michel et al. 2010; Nosil 2012; Riesch et al. 2017; Kopp et al. 2018). Factors that increase linkage disequilibrium include geographic structure, habitat or mate preferences, genetic architecture, and "magic traits" that produce both ecological divergence and assortative mating (Kirkpatrick and Ravigné 2002; Servedio et al. 2011; Flaxman et al. 2013, 2014; Nonaka et al. 2015; Kopp et al. 2018; Mendelson and Safran 2021). Social plasticity is an additional factor that may promote signal-preference co-divergence (cf. Bailey & Moore 2012; Rebar & Rodríguez 2015).

Here we focus on plasticity arising from interactions in mixed aggregations of diverging populations or recently diverged species, as in sympatric speciation or in allopatric speciation upon secondary contact with incomplete reproductive isolation. In such cases, plasticity in mate preferences or signals could arise from learning due to prior positive or negative experiences with potential mate types (e.g. Dukas 2004; Dukas et al. 2006; Rather et al. 2022); imprinting (Servedio et al. 2009; Hebets and Sullivan-Beckers 2010; Verzijden et al. 2012); or other effects of the social environment such as habituation or exposure to differing degrees of sexual trait variability (Bailey 2011; Bailey and Moore 2012; Fowler-Finn and Rodríguez 2012*a*, 2012*b*; Rodríguez et al. 2013*c*). The diverging populations might show various forms of plasticity in response to the different social environment caused by that contact (Fig. 1). They might respond in the same way, so that their phenotypes shift in similar directions and magnitudes (Fig. 1b-c). Alternatively, they might respond differently. The populations might become more similar to each other; e.g., if they were to imprint on each other (Fig. 1d). Such effects would be interesting, perhaps promoting the establishment of novel signals (cf. Broder et al. 2021; Tibbetts and Snell-Rood 2021), but would not enhance assortative mating. Here we note that the converse is also possible, however: the populations could become more different from each other (Fig. 1e), establishing or enhancing assortative mating. To our knowledge, this is a possibility that has not been explored. However, it arises intuitively from consideration of variation in the "sign" of the effects inducing plasticity. Additionally, there could be population and/or sex differences in the magnitude and direction of plasticity (Fig. 1f-g) with one population or sex lacking plasticity altogether but still contribute to assortative mating.

Here we test the hypothesis that plasticity in response to interactions in mixed-species aggregations creates or enhances signal-preference co-divergence (Fig. 1e-g). To do this, we tested for plasticity in signals and preferences generated by interactions between two members of the *Enchenopa binotata* species complex of treehoppers (Hemiptera: Membracidae). Species in this clade of host-specialist sap-feeding insects show remarkable signal-preference coevolution (Rodríguez et al. 2006; Cocroft et al. 2008, 2010; Rodríguez et al. 2013*c*). As many plant-feeding insects, *Enchenopa* communicate with plant-borne vibrational signals, both as juveniles and as adults (Cocroft and Rodríguez 2005; Cocroft et al. 2008; Hill 2008; Hill and Wessel 2016; Rodríguez et al. 2018; Rodríguez and Desjonquères 2019). These interactions are an important cause of plasticity in adult signals and preferences, with inputs from the social environment and developmental at different life stages having varying effects (Fowler-Finn and Rodríguez 2012a, 2012b; Rebar and Rodríguez 2013, 2014a, 2014b, 2015; Fowler-Finn et al. 2017; Desjonquères et al. 2019a, 2019b, 2021; Desjonquères & Rodríguez accepted). The strongest social effects on signals and preferences appear to result from the amount and nature of signalling interactions in the juvenile stage (Desjonquères et al. 2021; Desjonquères & Rodríguez accepted). Our two study species differ in their rate of signalling as juveniles (Rodríguez et al. 2018). Here we examine the overall effect of life-long interactions, by rearing individuals from nymphs to adults in treatments of mixed-species vs. own-species aggregations. We tested the effect of these treatments on the adult treehoppers' male advertisement signals, female preferred signal values (peak preferences), and on the strength of those preferences (Kilmer et al. 2017).

Our two species likely diverged from each other within the last 130-60 thousand years (Hsu et al. 2018). There is strong support for sympatric speciation across the *E. binotata* complex, driven by colonization of novel host plants and signal-preference coevolution (Wood 1993; Cocroft et al. 2008). For *E. binotata* populations on *Viburnum* host plants, however, there is evidence suggestive of a role for isolation by distance and/or secondary colonization from other hosts (Cocroft et al. 2010; Hsu et al. 2018). Thus, our two study species likely represent a case of recent secondary encounter on *V. lentago* in some of our study sites. Our geographic sampling (see below) also allows testing for reinforcement in signals and preferences and reinforcement in their plastic response to the treatment for the species that occurs both in sympatry and allopatry at our sites, as there has been an opportunity for selection against hybridization between our two study species at the sites where they co-occur.

## Material and methods

### *Field collection*

Most of the species in the *E. binotata* complex survive poorly on hosts used by other members of the complex (Wood and Guttman 1983; Cocroft et al. 2008). Reproductive isolation between species in the complex arises from multiple causes—phenological differences between host plants; physiological host specialization; behavioural host preferences; and behavioural mate preferences for species-specific advertisement signals (Wood 1993; Cocroft et al. 2008). Nevertheless, there are some sites throughout the range of the complex across North America where different *E. binotata* species live on the same host (Cocroft et al. 2010; R. B. Cocroft, pers. comm.). We took advantage of one such case in Wisconsin (USA), where two members of the complex occur on *Viburnum lentago* (Adoxaceae) plants at some sites (Rodríguez et al. 2018). Only one of our study species occurs at most sites in our study area, with the exception of two sites where both species co-occur reliably in distributions that have remained stable since we first observed them in 2012 (micro areas of the order of a few square meters within each site where either species occurs alone and micro areas where both occur side by side, sometimes on the same plant; D. Rebar & R. L. Rodríguez, unpubl.). Despite extensive searching, we have never found allopatric sites with the other species so all observations of it are from the two sympatric sites (see below).

Most members of the *E. binotata* complex have not been formally described (Hamilton and Cocroft 2009). However, they can be readily distinguished by their host plant species, nymph coloration, and adult signal frequencies (Pratt and Wood 1992; Cocroft et al. 2008). For shorthand, here we refer to our study species as $sp_{low}$ and $sp_{high}$ because of their distinctive male signal frequencies (ca. 165 and 275 Hz respectively) and corresponding female mate preferences (peak preferences at ca. 185 and 295 Hz respectively) (Rodríguez et al. 2013*b*; Rebar and Rodríguez 2015; Rodríguez et al. 2018).

We conducted the experiment over the summers of 2018-2020. Each June, we collected 3rd instar nymphs (the earliest stage at which the species can be distinguished by their different nymph coloration; Fig. 2; Rodríguez et al. 2018b) from five populations (Fig. S1). These sites include three allopatric sites with only $sp_{low}$ (BOG, OLT and PNV) and two sympatric sites with both $sp_{low}$ and $sp_{high}$ (FST and FGC). We collected more than 3200 individuals during those three years (Table S1) that were subsequently installed on rearing plants at the University of Wisconsin-Milwaukee greenhouse (total of 80 plants over the three years).

### *Rearing aggregation treatments*

We created treatments consisting of own- or mixed-species aggregations (Fig. 2-3). We randomly assigned individuals from each of the above sites to one or the other treatment. To assemble an aggregation, we placed 40 nymphs on a 40-60 cm-tall potted *V. lentago* host plant. In the own-species treatment, each rearing plant/aggregation either contained 40 $sp_{low}$ or 40 $sp_{high}$ nymphs from a single site. In the mixed-species treatment each rearing plant/aggregation had 20 $sp_{low}$ and 20 $sp_{high}$ nymphs (Fig. 3). Within species, all individuals were from the same site but the two sets of 20 individuals in the mixed-species treatment could be from either the same or different sites.

Although species in the *E. binotata* complex can be distinguished as nymphs by their distinctive coloration patterns ($sp_{low}$ nymphs are grey, whilst $sp_{high}$ are dark brown with white stripes; Fig. 2; Rodríguez et al. 2018), the adults are very similar morphologically (Pratt and Wood 1992; Cocroft et al. 2008). In order to distinguish the adults in our experiment, we marked them once they moulted with white or red non-toxic acrylic paint (Apple Barrel matte acrylic paint 21469E Flag Red and 20503E White; Plaid Norcross, GA, USA). This required a brief separation of late instar nymphs in the mixed-species groups (Fig. 3). When the very first adults moulted in the mixed-species groups, we separated the remaining nymphs from each replicate onto two separate plants—one for each species (we excluded those first few adults which moulted from the experiment, as we had no way of knowing their species identity).Then, as new adults moulted, we marked them and re-assembled the experimental aggregations. We applied exactly the same manipulation to own-species aggregations (separation and marking) to avoid confounding effects. Thus, individuals spent 7.1 ± 3.8 days (mean ± SD) out of their treatments. At that time in the life of these insects, there is little signalling by nymphs (Desjonquères et al. 2019*a*) and adult signalling has not yet begun (see below), so this represented little interruption in the experimental manipulation. In the re-assembled aggregations of adults, we also separated the sexes onto different plants to prevent females from mating and becoming unresponsive to playbacks (see below). We randomly switched the colour for species across plant replicates to avoid confounding effects of colour. For own-species replicates, we randomly assigned a colour to each plant aggregation, such that about half were white and half were red.

### *Adult signals recording and analysis*

We recorded adult male and female vibrational signals with laser vibrometry. We focused a portable laser Doppler vibrometer (Polytec PDV-100; Polytec Inc. Auburn, MA, USA) on a piece of adhesive reflective tape on the stem of the recording plant (a potted *V. lentago* plant). The signal was band pass filtered between 40 and 3000 Hz with an electronic variable filter (model 3202; Krohn-Hite, Brockton, MA, USA) and transferred to an iMac computer (MacBook Pro; Apple, Cupertino, CA, USA) with a USB audio interface (Edirol USB Audio Capture UA-25; Roland, Hamamatsu, Japan). We recorded the output on the iMac with the program AUDACITY (v. 2.1.2; http://audacity.sourceforge.net/) at a sampling rate of 44.1 Hz. We used two digital thermometers (Fisher scientific, Pittsburgh, PA, USA; and Extech Instruments SDL500, Nashua, NH, USA) to monitor room temperature during signal and preference recordings.

Sexually active *Enchenopa* males signal spontaneously when placed on a stem of their host plant (starting approximately two weeks after the adult moult). We placed each male on the recording plant and if the male did not signal after one minute, we primed the male with a playback of a recorded male followed by a female response (see below for vibrational playback method). To avoid making assumptions about the signal phenotypes resulting from the experiment, we primed all males with both $sp_{low}$ and $sp_{high}$ playbacks, emitting one primer of each species separated by 30 seconds of silent interval for a total of two primers in random order. If a male did not signal within 10 minutes, we placed him back on his replicate plant and tried again every 2-4 days or until he died. Males that signalled did so within 2.8 ± 1.6 tries (mean ± SD). From the resulting 150 male recordings (Table S1), we measured the dominant frequency of male signals using AUDACITY and core functions in R (V3.0.6; R Core Team 2015).

### *Female mate preference description*

Sexually receptive *Enchenopa* females (starting approximately four weeks after the adult moult) duet with the signals of males that they find attractive, providing a realistic, natural, and convenient means for assessing their mate preferences for male signal traits (Rodríguez et al. 2004, 2006, 2012; Cocroft et al. 2008). To describe female preferences for signal frequency, we presented vibrational playback stimuli through a piezoelectric stack coupled to the stem of the plant with soft wax, driven by a piezoelectric controller (Thorlabs, Newton, NJ, U.S.A.). We recorded female signals and playbacks with the laser vibrometer as described above. The amplitude of playback stimuli was calibrated to 0.15 mm/s using an oscilloscope. We placed each female on a potted plant, allowing her to settle for 30 seconds, we then tested whether the female was receptive with a maximum of 6 primer playbacks of recorded male signals from both species. The $sp_{low}$ and $sp_{high}$ primer playbacks were emitted in alternation and separated by 15 seconds of silent intervals. If a female did not respond to any of the 6 primers, we returned her to her replicate plant and tried again every 2-4 days or until she died. If the female responded to a $sp_{low}$ (or $sp_{high}$) primer, we gave her a full preference sequence. The 374 females (Table S1) that responded did so within 1.9 ± 1.4 tries (mean ± SD).

To obtain female preference functions, we used vibrational playback sequences composed of synthetic stimuli varying in frequency, with all other features set to the population mean of each species (e.g., $sp_{low}$ males produce signals with 4 pulses/signal, so each of our stimuli had 4 pulses per signal, and so on; see Table S2 for details about the stimuli features). We exposed each female to a randomized sequence of 18 playback stimuli. To capture the full shape of the preference functions, the range of stimuli frequencies varied from 100 to 440 Hz in 20 Hz increments, exceeding the range of signal frequency values in the two species (Kilmer et al. 2017). Each playback stimulus was a bout with four signals with that frequency, each separated from the next by 1.9 or 2.5 sec (for $sp_{low}$ and $sp_{high}$ respectively, based on average population values) of silence. Each playback bout was separated from the next by 15 sec. of silence. We assayed female preference with the number of responses (between 0 if she did not respond to any signal and 4 if she responded to all the signals in the synthetic bout) that each female produced in response to each of the 18 stimuli. A score of 4 responses for a stimulus thus indicates maximum attractiveness, and a score of 0 the lowest attractiveness.

We fitted cubic spline regressions to the response data for each female and generated individual preference function curves using the program *PFunc* (v. 1.0.0; https://github.com/Joccalor/PFunc and https://hub.docker.com/r/joccalor/pfunc/; Kilmer et al. 2017). This approach allows any shape for the preference functions with a certain level of smoothness that is determined empirically (Schluter 1988; Kilmer et al. 2017). *PFunc* fits curves using the gam function in the *mgcv* R package (Wood and Wood 2015). We used the default smoothing parameter values calculated by *PFunc* for all our curves, setting the range of smoothing values between 0.005 and 0.5. This means that females could vary in smoothing values set for their curve (see Table S3 for all smoothing values). Additionally, we checked all curves and slightly changed the smoothing value for females with curves that strongly deviated from the raw data (Fig. S2). Smoothing values did not differ significantly between species and treatment (linear model with smoothing as a response variable and species, treatment, and their interaction as test variables; p-value>0.45 for the three terms). We then analysed variation in the individual preference functions using the preference peak and preference strength metrics implemented in PFunc (Kilmer et al. 2017). Preference peak is preferred display trait value, measured as the signal frequency with the highest response likelihood on the preference function. Preference strength is the degree to which attractiveness falls away from peak preference, calculated as [SD(response values)/mean(response values)]$^2$ where SD=standard deviation. These two traits are highly repeatable (peak preference: $r$ = 0.40; preference strength: $r$ = 0.76; Cirino et al. in review).

### *Statistical analysis*

We conducted all analyses using the *lmer* function of the R-package *lme4* (v. 1.1-25; Bates et al. 2014). We built linear mixed models (described below) in which the error structure was Gaussian. We checked the assumptions of normality and homoscedasticity of residuals by visually examining a quantile-quantile plot and the residuals against the fitted values, both indicating no deviation from these assumptions. We assessed model stability by excluding data points one at a time from the data, fitting the model and collecting the parameter estimations. If the range of parameter estimations over all these iterations included 0, the model and variable estimation was considered unstable. To test for collinearity between fixed effects, we derived variance inflation factors (Field 2009) using the function vif of the R-package car (version 2.1-4; Fox & Weisberg 2011) and they revealed the absence of collinearity between fixed effects (maximum value of 1.5; collinearity issues usually indicated by values higher than 4).

#### Testing for plasticity due to rearing in own-species vs. mixed-species aggregations

To test for an effect of the rearing treatments on male signal frequency and female peak preference, we built a linear mixed model in which we used a "reaction norm" approach with one dependent variable that represented both male signal frequency and female peak preference (cf. Fowler-Finn et al. 2015; Rebar and Rodríguez 2015). This approach allowed us to analyse the relationship between the effects of the treatments on both preferences and signals with a single model. The model had the following explanatory variables: treatment (“mixed” or “own”), species ($sp_{low}$ or $sp_{high}$), sex (male or female), year (categorical variable with three levels: 2018, 2019 or 2020), and recording temperature. Recording temperature and year were included as control variables. Typically, year would be included as a random factor, but because it had less than five categorical levels, we included it as a fixed effect (Arnqvist 2020). The model also included all two- and three-way interactions between treatment, species, and sex. These interactions terms test for test for species and sex differences in the plastic response to the rearing treatments. For instance, the best-case scenario for plasticity arising from interactions in mixed species aggregations to contribute to assortative mating would require that the signals and preferences of each species become more distinct in the mixed treatment (Fig. 1e). This would be indicated by a significant species × treatment interaction (with visual inspection to distinguish between the scenarios in Fig. 1d vs. e). Other scenarios would be indicated as follows: no significant effects (Fig. 1a); only treatment significant (Fig. 1b,c); significant sex × treatment interaction and species × treatment and/or 3-way interaction (Fig. 1f,g). As there were several individuals on each rearing plant/aggregation, the model also included rearing plant/aggregation identity as a random term. We initially included collection site as a term, but it was never significant ($p>0.07$ in all cases), so we removed it from our analyses.We used a second, similar model to test for an effect of the treatments on female preference strength, with preference strength as the dependent variable.

### Testing for reinforcement in $sp_{low}$

We focused this analysis on $sp_{low}$, for which we had both sympatric and allopatric populations (we were unable to find allopatric sites for $sp_{high}$, despite considerable efforts; see above). We built a linear mixed model with frequency (of male signals or female preference) as the dependent variable. The explanatory variables were: treatment, population type (allopatric or sympatric), sex, year and recording temperature. We included an interaction between treatment and sex to keep the model as similar to the previous one as possible to keep them comparable. We also included an interaction between treatment and population type to test for differences in the effect of treatment in different population types. The model included rearing plant/aggregation identity as a random term. We used a similar model to test for geographic variation in the form of plasticity for female preference strength in $sp_{low}$.

## Results

### *Plasticity due to rearing in own- vs. mixed-species aggregations*

Our study species responded differently to the rearing treatments of own vs. mixed-species aggregations (significant species × treatment, and species × treatment × sex interactions; Table 1), supporting a scenario similar to Fig. 1f-g. $Sp_{high}$ individuals reared in mixed-species aggregations differed more (by 11% on average) from $sp_{low}$ in male advertisement signals and female mate preferences than individuals reared in own-species aggregations (Fig. 4, 5, and 6a-b). By contrast, $sp_{low}$ exhibited little plasticity in signals or preferences in response to the rearing treatments (Fig. 4, 5, and 6a-b). Note, however, that $sp_{low}$ and $sp_{high}$ showed comparable overall plasticity in signals and preferences due to within-treatment variation in developmental and social environments (significant random terms for rearing plant/aggregation; Table 1). Interestingly, the variance in male signals did not differ between treatments while the variance in female peak preference was higher in own- than in mixed-species treatments for both species (Table S4).

The rearing treatments also tended to affect female preference strength differently in the two species (marginally significant species × treatment interaction, Table 2). $sp_{high}$ females reared in mixed-species aggregations tended to have stronger preferences than females reared in own-species aggregations (Fig. 6c). By contrast, $sp_{low}$ females exhibited little plasticity in preference strength according to the rearing treatments but tended to express constitutively higher strength than that of $sp_{high}$ females in own-species aggregations (Fig. 6c). Nevertheless, as above, both species showed comparable plasticity due to within-treatment variation in the developmental and social environment (significant random terms for rearing plant/aggregation; Table 2).

### *No reinforcement in* $\mathrm{sp}_{\mathrm{low}}$

There was no difference between sympatric and allopatric populations in signals and preferences within treatment (non-significant population type; Table S5; Fig. 4, 5, and 6a-b) and in the form of the plastic response to the rearing treatments in signals or preferences (non-significant population type × treatment interaction; Table S5; Fig. 4, 5, and 6a-b). There was also no difference between sympatric and allopatric populations in female preference strength within treatment (non-significant population type; Table S6; Fig. 6c) and in the form of the plastic response in female preference strength (non-significant effect of population type × treatment interaction; Table S6; Fig. 6c).

## Discussion

Here, wee propose a heuristic model whereby plasticity due to interactions in mixed aggregations of diverging populations or recently diverged species may create or enhance signal-preference differences and promote assortative mating (Fig. 1). We also present a "proof of concept" test of this hypothesis with two recently diverged species in the *E. binotata* complex of treehoppers that differ in signals and preferences but not ecologically. We found that social plasticity enhances signal-preference differences between two closely related species of *Enchenopa* treehoppers mediated via the plastic response of one species. When reared in mixed-species aggregations, males of $sp_{high}$ had higher frequency signals and females had preferences for higher signal frequencies than when reared in own-species aggregations. The other species ($sp_{low}$) did not show plasticity in response to these rearing treatments.

Social plasticity enhanced signal-preference differences between these two species by about 10%. This is a relatively small, but important increase in the signal-preference species differences. Females *E. binotata* have strong preferences for signal frequency, and a 10% deviation from peak preference typically decreases signal attractiveness by ca. 50% (Rodríguez et al. 2006, 2013*a*). Further, the combined effect through signals and preferences further enhances that effect. Despite a wide frequency gap between the two species, the range of variation in the population, especially in female preferences (Fig. 4), points to some risk of hybridization. Indeed, some females appear potentially willing to accept males of the other species (e.g. female 12, 25, 33, 260, 289 or 291 in Fig. S2). Hence, we consider that the observed plastic response is likely to increase assortative mating in a biologically relevant way between the two species. These results support a key component of the scenario outlined in Fig. 1g: a species difference in social plasticity with plasticity of the predicted sign in one species and no plasticity in the other species.

Mate preferences can differ in preferred signal value, but also in strength, and the two can have distinct evolutionary consequences (Bailey 2008; Rodríguez et al. 2013*a*; Kilmer et al. 2017). Our rearing treatment not only influenced phenotypic values for male signals and female peak preferences, but also the strength of those preferences: $sp_{high}$ females reared in mixed-species aggregations had stronger preferences than those reared in own-species aggregations. This compounded effect could further increase assortative mating when the two species are in contact, as females not only have a preference for higher frequencies, but that preference for higher frequencies is stronger. $Sp_{low}$ female preference strength was not plastic in response to our treatments, but constitutively higher than that of $sp_{high}$ females in own-species aggregations. The combined effect of preference peak and strength thus results in a reduction of $sp_{low}$ signal attractiveness for $sp_{high}$

females, likely further enhancing assortative mating.

Although we did not attempt to identify the specific inputs into trait expression that may be responsible for the observed plasticity, several aspects of social interactions may have been at play. The manipulation in the social environment that we applied resulted in lifelong changes in the interactions experienced by individuals. As juveniles, $sp_{low}$ have higher signalling rates than $sp_{high}$ (Rodríguez et al. 2018). Thus, the mixed treatment likely presented a change for nymphs of both species, with higher than usual signalling rates for $sp_{high}$ and lower than usual for $sp_{low}$. Males of the two species differ in their advertisement signals (mainly in dominant frequency, see above), and that would offer strong differences in experience between the treatments. However, prior work found that such differences in male adult experience alone do not change male signal frequency (Rebar and Rodríguez 2016). Further, although the strongest effects found were on female mate preferences, females were not exposed to male signals during the treatments and would not themselves signal until later in life and then mainly in response to males, so our treatments likely varied little at this stage for females. Consequently, we consider that our results likely arise from the effects of inputs that occurred during the juvenile stage, which prior work has shown to be important (Desjonquères et al. 2019*b*, 2019*a*, 2021).

The observed divergence-enhancing plasticity could arise in two ways. It could occur upon first encounter—without prior selection against hybridization—and immediately establish or strengthen assortative mating. Alternatively, it may arise from selection against hybridization—i.e., reinforcement (Servedio and Noor 2003). Reinforcement could act not only on signals or preferences themselves, but also on their plastic response (Lesna and Sabelis 1999; Pfennig 2007; Chaine and Lyon 2008). The "first encounter" and "reinforcement of plasticity" scenarios may be contrasted by testing for geographic variation in the form of plasticity and measuring the fitness of hybrids. Under the "reinforcement of plasticity" scenario, plasticity due to interactions in mixed-species aggregations would create or enhance signal-preference differences only in individuals from sympatric populations where both populations or species have a history of co-existence, and not in individuals from allopatric sites where only one species occurs. We occasionally find treehoppers with intermediate signals and preferences (a few individuals out of hundreds collected each year; K. D. Fowler-Finn & R. L. Rodríguez, unpubl.), suggesting that these species may hybridize at low rates in the field. However, hybrids are unlikely to mate, as their intermediate signals and preferences will fail to be attractive to (or be attracted by) either parental species. We found no differences in the plastic response between sympatric and allopatric sites for $sp_{low}$, but we were unable to conduct a similar test for $sp_{high}$. Future work would profit from a renewed population sampling effort to confidently test a "first encounter" versus "reinforcement" scenario.

Our results support the hypothesis that social plasticity can create or enhance signal-preference differences and promote assortative mating. Specifically, we suggest that a change in the social environment can enhance phenotypic differences in mating signals and mate preferences, promoting reproductive isolation. This process might represent an under-appreciated cause of assortative mating and signal-preference divergence in the early stages of speciation. Once present, new or enhanced signal-preference differences expressed due to social plasticity would not only promote assortative mating, but also facilitate further co-divergence through subsequent evolution of signals, preferences, and/or their plastic response (the latter potentially involving genetic accommodation or assimilation; West-Eberhard 2003, 2005). Such subsequent evolution may lead to genetic change in signals, preferences and/or the machinery involved in their development, as well as genetic change in the elements of the social environment responsible for the plasticity-inducing inputs—change in the indirect genetic components of signals, preferences and their developmental regulation (cf. Bailey & Moore 2012; Rebar & Rodríguez 2015). The importance of this process for speciation will depend on how common, how strong, and of what sign, the "first encounter" effects of social plasticity are. Further, reinforcement of the plastic response is an interesting and potentially important outcome that should be explored further with experimental research. Such early unselected, “first encounter” plasticity may even contribute to that reinforcement through genetic accommodation of the plastic response. Comparative work to answer these questions and test these hypotheses will be illuminating.

## Acknowledgements

We thank Paul Engevold for his assistance and greenhouse expertise, and the University of Wisconsin–Milwaukee Field Station staff for their support. We also thank Vladislav Melnikov, Nour Abuomar, Bernard Muransky, and Nathaniel Chester Wagner for their help with insect and data collection. Finally, we thank Mike Ritchie and three anonymous reviewers for his insightful comments on the manuscript. We acknowledge the support of the Fondation Fyssen (to CD), the Research Growth Initiative from University of Wisconsin- Milwaukee (to RLR), the National Science Foundation (Grant IOS−1855962 to RLR and CD), and the Natural Environment Research Council (grants NE/T000619/1 and NE/W001616/1 to NWB). We declare that we have no competing interests.

## Statement of authorship

CD and RLR obtained the funding to run the experiments. CD, GH, NWB and RLR came up with the hypotheses. CD, BS, SS, CS, JM, and CW ran the experiments under the supervision of CD and RLR. CD, LAC, and IE extracted the data from the recordings. CD and RLR worked on the representation of the data and wrote the original draft. All authors contributed critically to the drafts and gave final approval for submission.

## Data accessibility statement:

The data related to this manuscript is available publicly on Zenodo (https://doi.org/10.5281/zenodo.7844980).

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## Tables

**Table 1: Variation in male signal frequency and female peak preference in two *Enchenopa* species according to own- vs. mixed-species rearing treatments.** Significant effects and marginally significant in bold.

| **Fixed effects** | | | |
|---|---|---|---|
| **Term** | $\chi^2$ | **Df** | **P-value** |
| Species | 4520.22 | 1 | **<0.0001** |
| Sex | 22.79 | 1 | **<0.0001** |
| Treatment | 2.01 | 1 | 0.16 |
| Year | 9.61 | 2 | **0.009** |
| Temperature | 14.67 | 1 | **0.00013** |
| Species × sex | 0.57 | 1 | 0.45 |
| Species × treatment | 5.10 | 1 | **0.024** |
| Sex × treatment | 0.14 | 1 | 0.71 |
| Species × sex × treatment | 5.70 | 1 | **0.017** |
| **Random effect** | | | |
| **Term** | $\chi^2$ | **Df** | **P-value** |
| Rearing plant/aggregation | 4.11 | 1 | **0.043** |

**Table 2: Variation in female preference strength in two *Enchenopa* species according to own- vs. mixed-species rearing treatments.** Significant and marginally-significant effects in bold.

| **Fixed effects** | | | |
|---|---|---|---|
| **Term** | $\square^2$ | **Df** | **P-value** |
| Species | 0.10 | 1 | 0.76 |
| Treatment | 0.17 | 1 | 0.68 |
| Year | 3.34 | 2 | 0.19 |
| Temperature | 0.54 | 1 | 0.46 |
| Species × treatment | 3.23 | 1 | **0.073** |
| **Random effects** | | | |
| **Term** | $\square^2$ | **Df** | **P-value** |
| Rearing aggregation | 4.30 | 1 | **0.038** |

## Figures

**Figure 1: Sketch of possible effects of social plasticity on signal-preference differences.** When two populations or closely related species first meet (a), interactions may increase or decrease the signal-preference values of both populations or species (b or c, respectively), make each population or species more similar to the other (d), or create/enhance signal-preference differences (e-g). There are many other possible scenarios combining species and sex differences in the plastic response (64 possible scenarios if we consider that each species' trait values could shift in 8 different directions). In the interest of space, we only illustrate the major categories of scenarios.

**Figure 2: Examples of own- and mixed-species rearing aggregations that constituted the treatments in our experiment.**

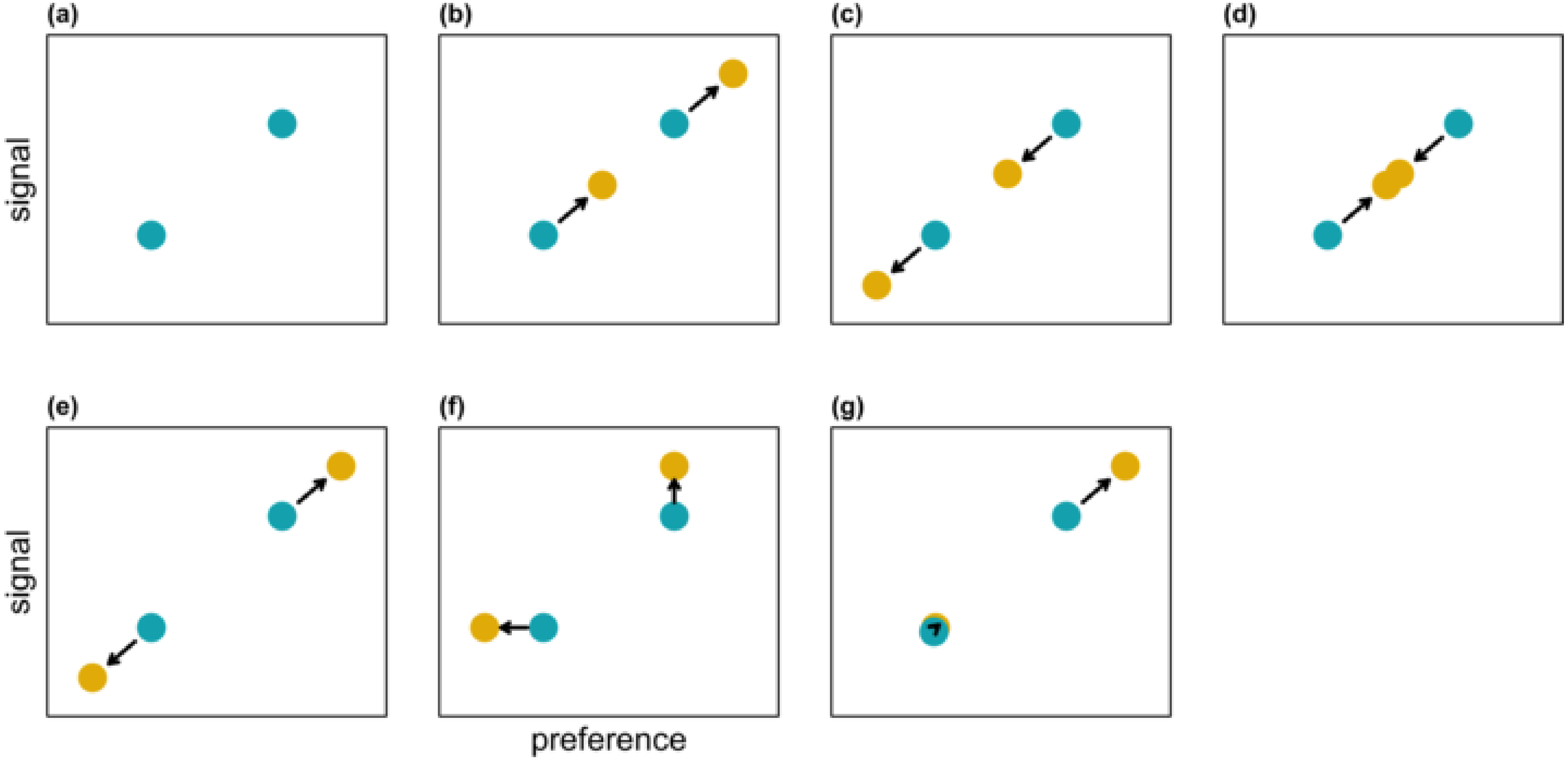
**Figure 3: Flowchart of the experimental design to rear individuals in own- or mixed-species treatments**. Nymphs were brought into a greenhouse and installed on host plants either as own- or mixed-species aggregations. When the first adults appeared, we separated the two species onto two rearing plants (for the own-species treatment, aggregations were just split on two new rearing plants to follow the same procedure as for mixed-species treatment). As adults continued to appear, we marked them according to their rearing plant and separated males and females.

**Figure 4: Variation in female preference curves in *Enchenopa* according to species and own- vs. mixed-species rearing treatments.** Dotted lines present individual-level preference curves. Solid lines present group-level preference curves for each treatment-species combination. Light blue shows females reared in own-species treatments and orange indicates those reared in mixed-species treatments. Left panel shows $sp_{low}$ and the right $sp_{high}$.

**Figure 5: The effect of own- vs. mixed-species treatments on male signal frequency and female peak preference in two *Enchenopa* species.** Points and associated bars show the mean and standard error for each rearing treatment–site–species combination (data corrected for the effects of temperature, year and plant replicate using model predictions). For reference, data from $sp_{low}$ and $sp_{high}$ species occupy the lower left and upper right portions of the plot, respectively. Blue shades

show means of individuals reared in own-species treatments and red and orange shades show means of individuals reared in mixed-species aggregations. Lighter colours (orange and light blue) are for individuals from sympatric populations while darker colours (red and dark blue) are for individuals from allopatric populations. There were no allopatric populations for $sp_{high}$ thus there are no dark colours for that species.

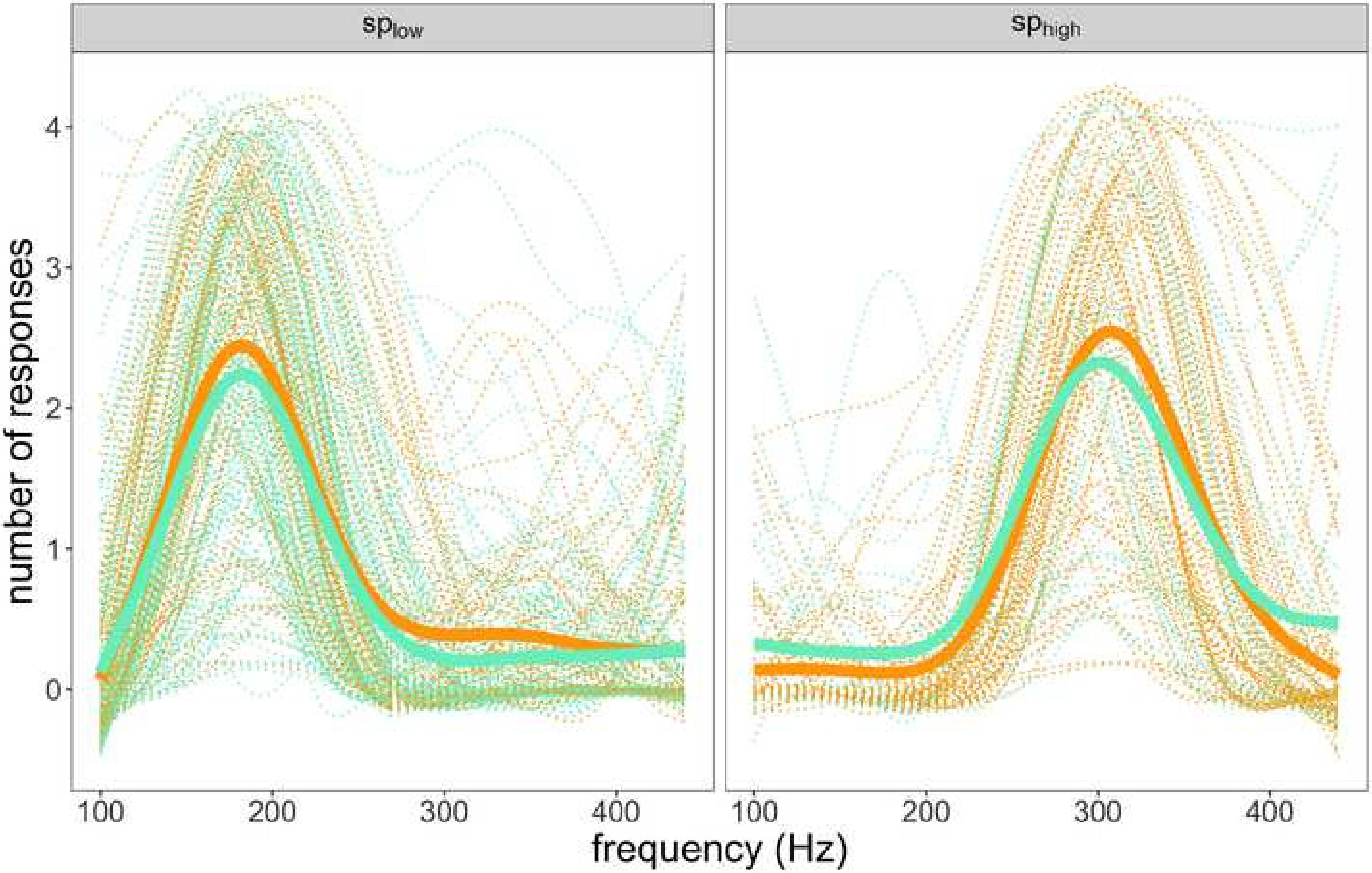
**Figure 6: The effect of own- vs. mixed-species treatments on male signal frequency (a), female peak preference (b) and preference strength (c) in two *Enchenopa* according to own- vs. mixed-species rearing treatments.** Solid points and associated bars show the mean and standard error for each rearing site–treatment–species combination (data corrected for the effects of temperature, year and plant replicate using model predictions). Transparent points show the individual data. Blue shades indicate means for individuals reared in own-species treatments. In red and orange shades indicate means for mixed-species aggregations. Lighter colours (orange and light blue) indicate individuals coming from sympatric populations while darker colours (red and dark blue) indicate individuals from allopatric populations. Note there were no allopatric $sp_{high}$ populations thus there are no dark colours for that species. Dotted grey lines show the reaction norm for each site (two sites for $sp_{high}$ and five sites for $sp_{low}$).

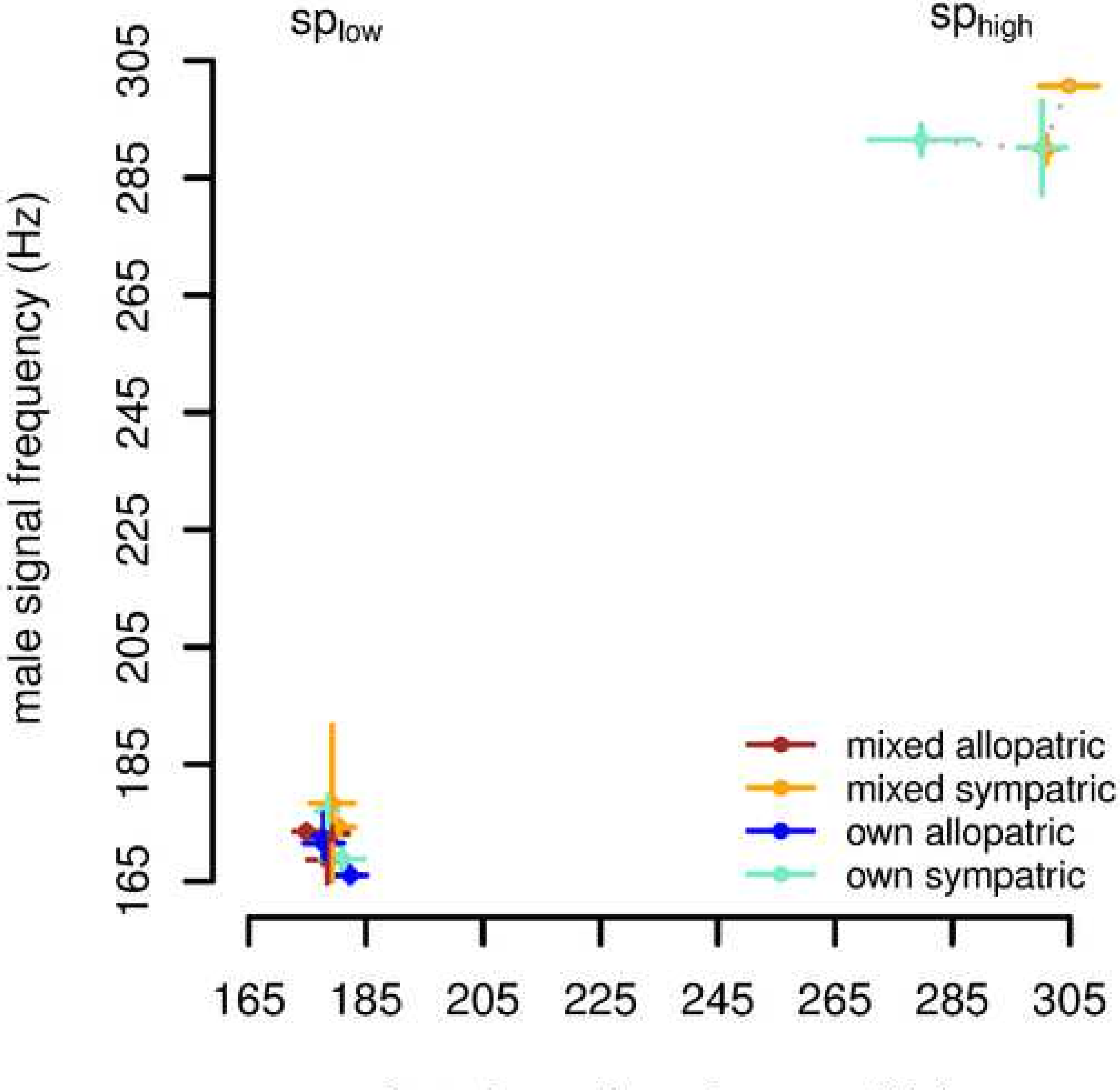

(a)
(b)
(c)
(d)
(e)
(f)
(g)
signal
signal
preference

(a) own, $sp_{low}$

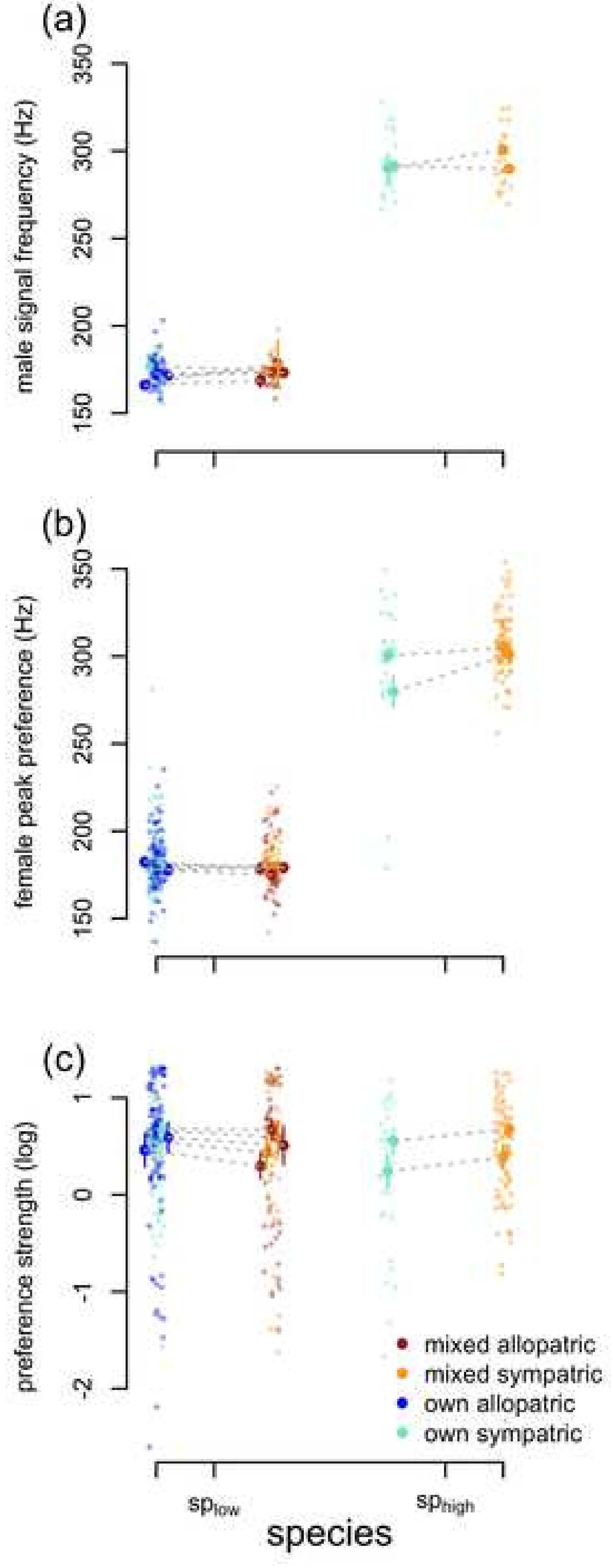
(b) mixed, $sp_{low}$ and $sp_{high}$

(c) own, $sp_{high}$

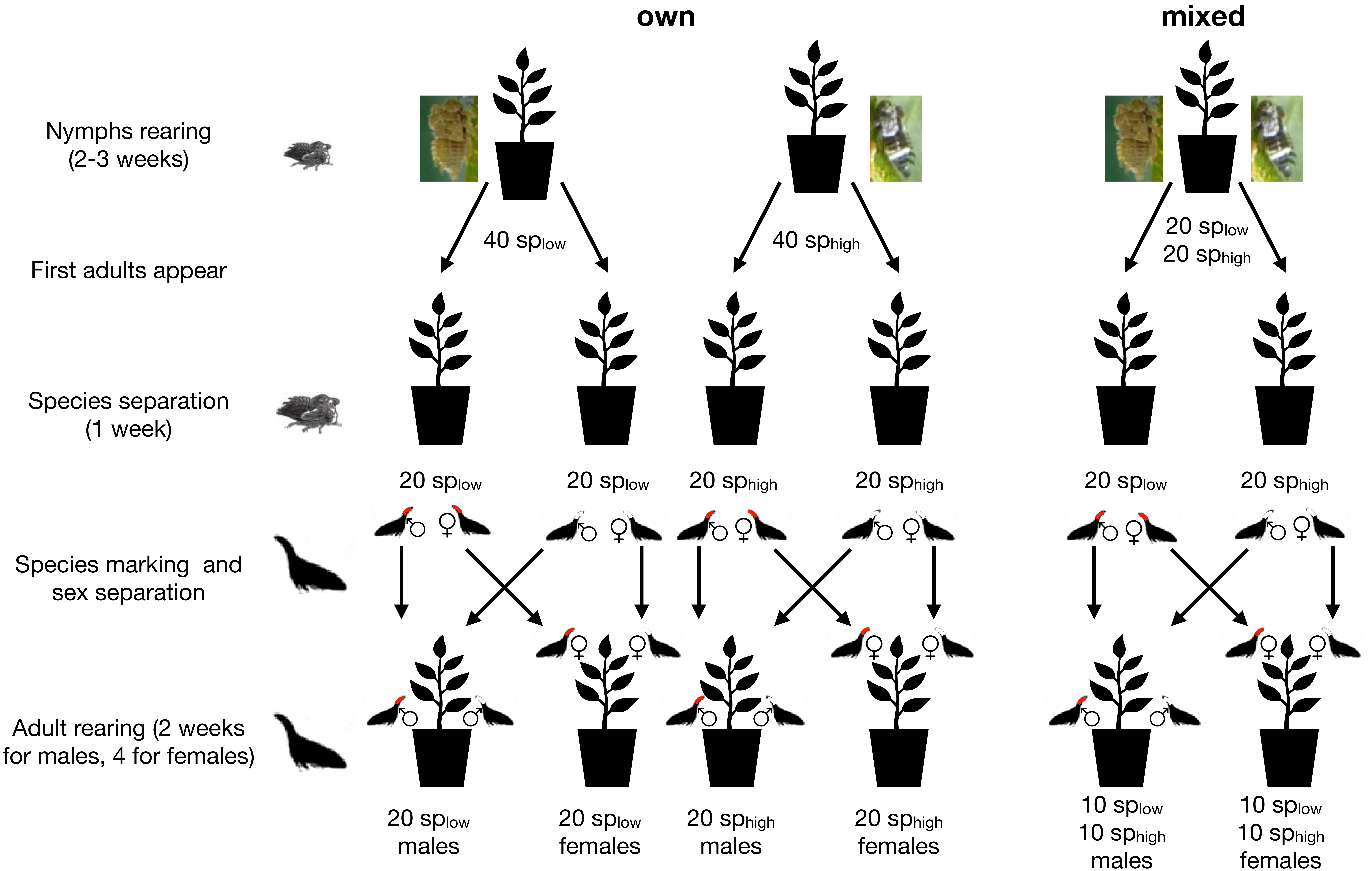

$sp_{low}$
$sp_{high}$
number of responses
0
1
2
3
4
100
200
300
400
frequency (Hz)



$sp_{low}$
$sp_{high}$
male signal frequency (Hz)
305
285
265
245
225
205
185
165
female peak preference (Hz)
165
185
205
225
245
265
285
305
mixed allopatric
mixed sympatric
own allopatric
own sympatric



(a)
male signal frequency (Hz)
350
300
250
200
150
(b)
female peak preference (Hz)
350
300
250
200
150
(c)
preference strength (log)
1
0
-1
-2
● mixed allopatric
● mixed sympatric
● own allopatric
● own sympatric
sp$_{low}$
sp$_{high}$
species


own
mixed
Nymphs rearing (2-3 weeks)
First adults appear
Species separation (1 week)
Species marking and sex separation
Adult rearing (2 weeks for males, 4 for females)
40 $sp_{low}$
40 $sp_{high}$
20 $sp_{low}$ 20 $sp_{high}$
20 $sp_{low}$
20 $sp_{low}$
20 $sp_{high}$
20 $sp_{high}$
20 $sp_{low}$
20 $sp_{high}$
♂ ♀
20 $sp_{low}$ males
20 $sp_{low}$ females
20 $sp_{high}$ males
20 $sp_{high}$ females
10 $sp_{low}$ 10 $sp_{high}$ males
10 $sp_{low}$ 10 $sp_{high}$ females

# Social plasticity enhances signal-preference co-divergence

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[2] School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, UK

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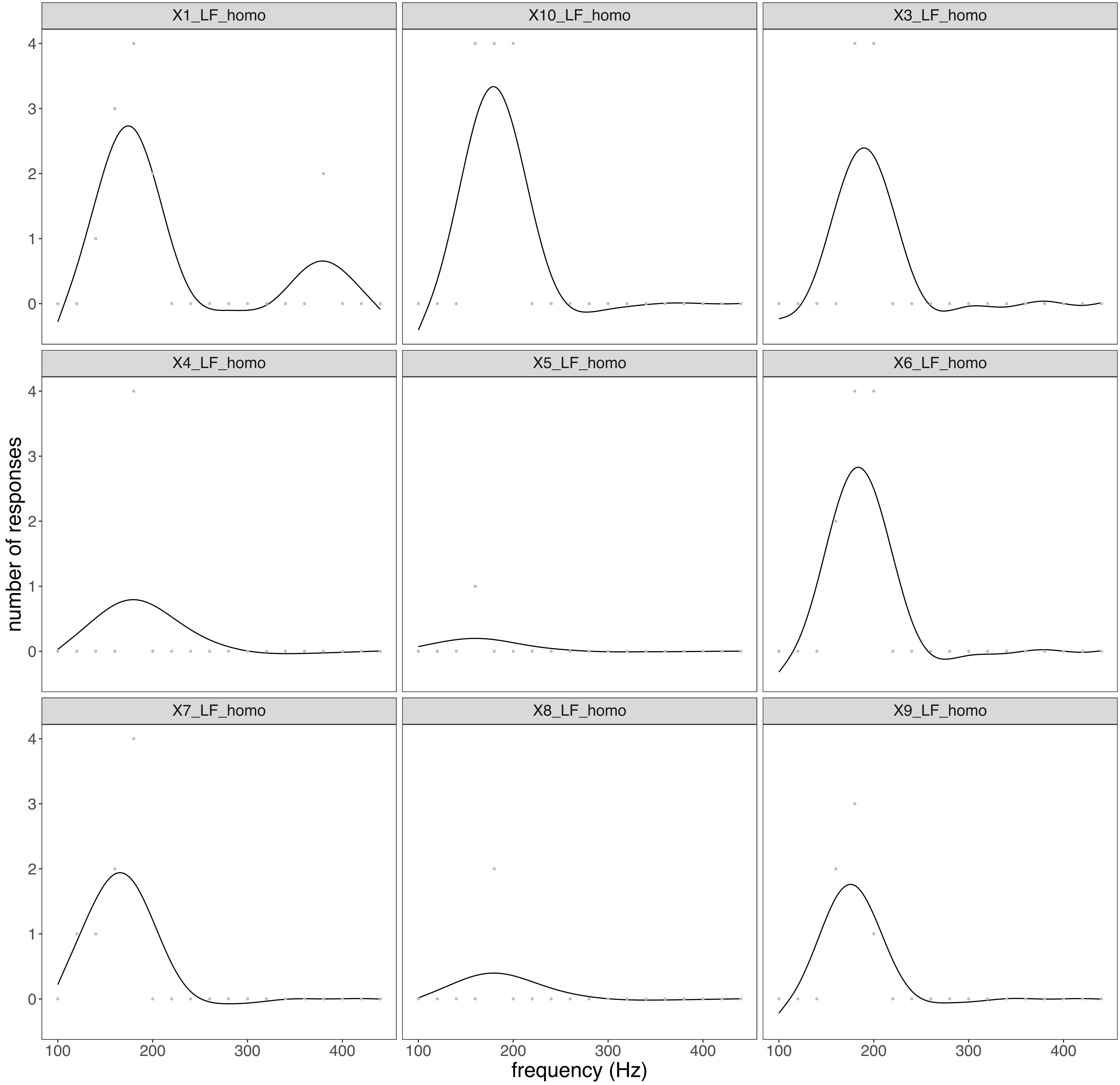
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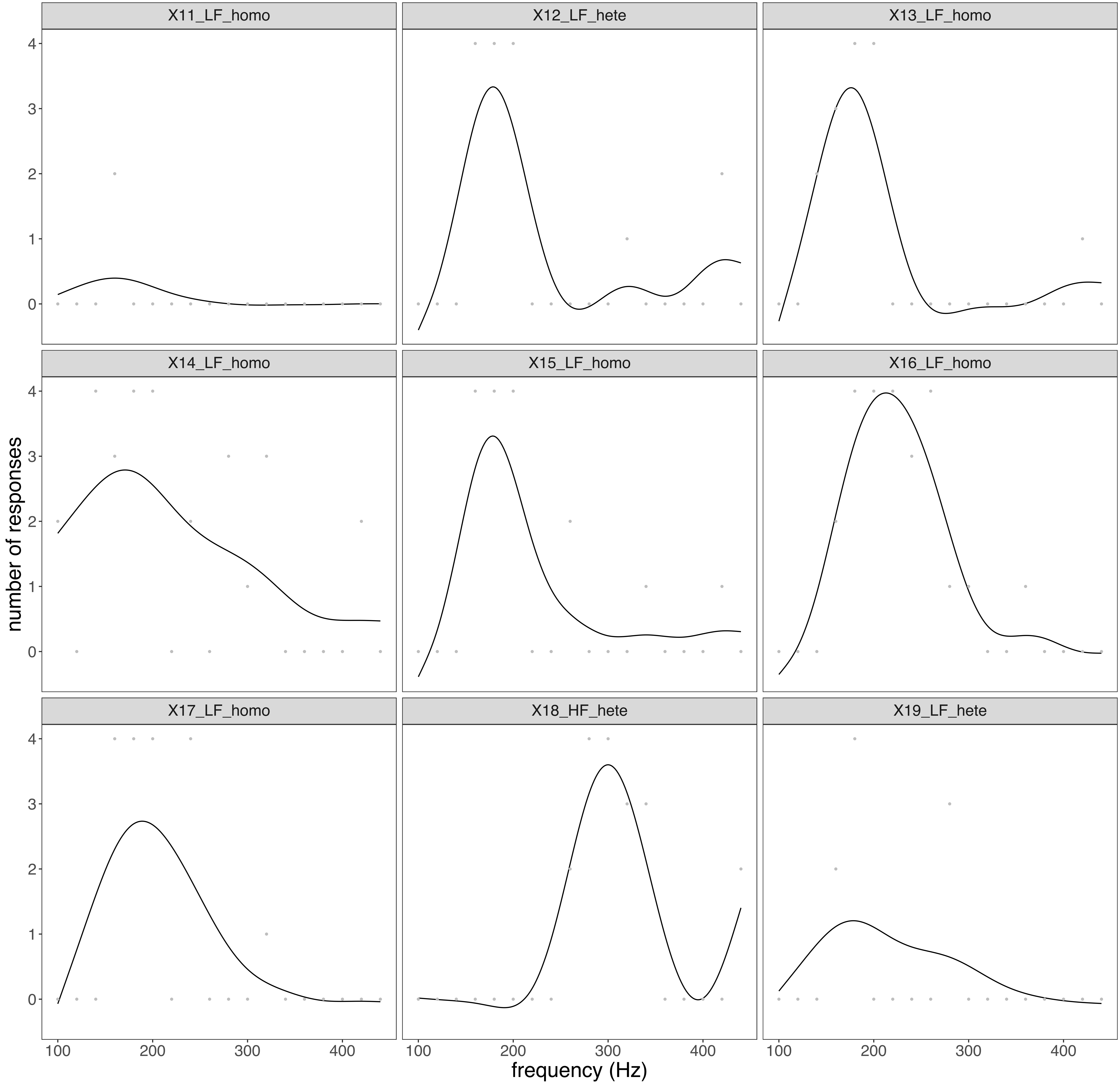
X9_LF_homo
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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4
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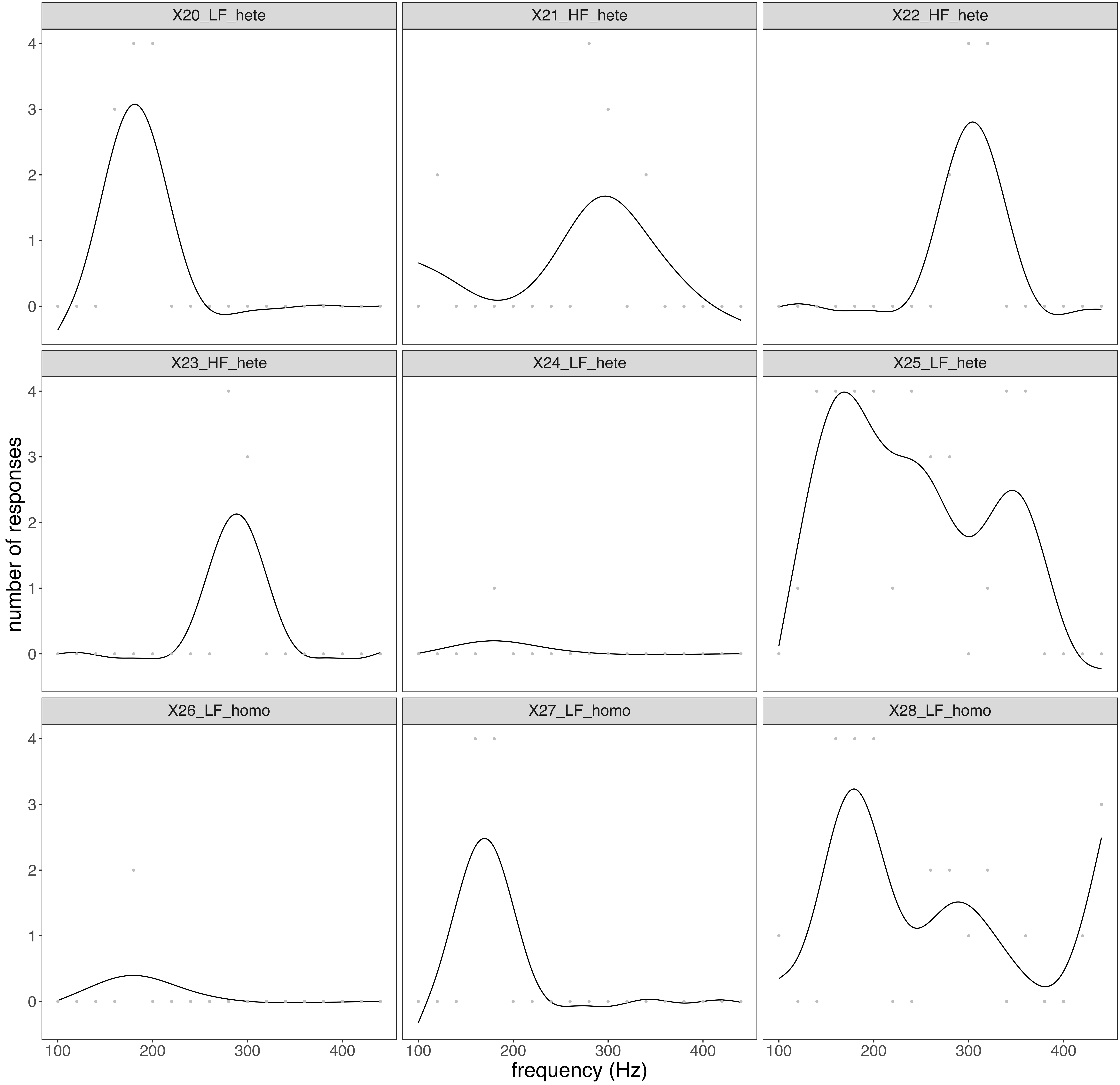
300
400
frequency (Hz)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2
3
4
frequency (Hz)
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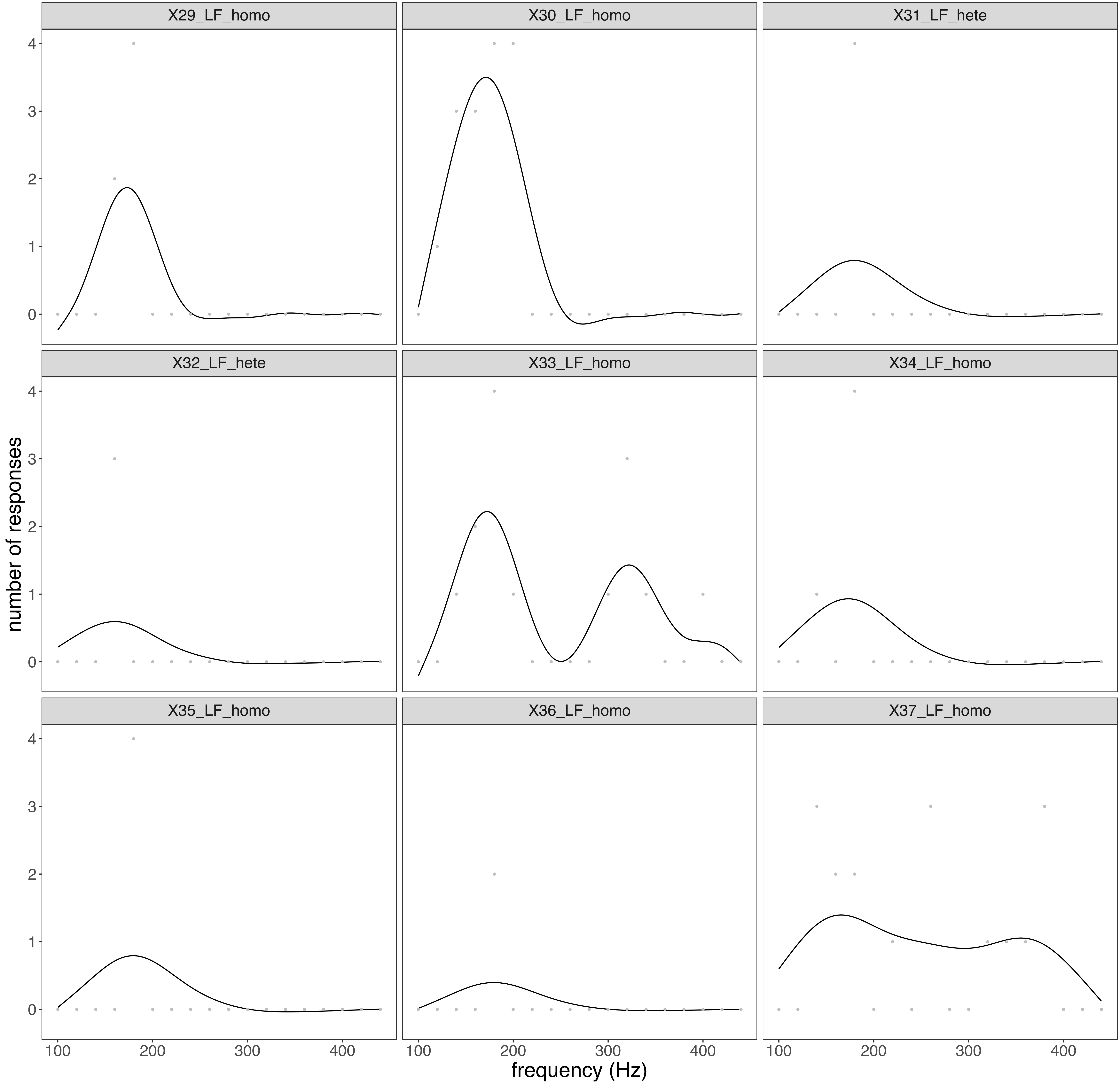
200
300
400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frequency (Hz)
100
200
300
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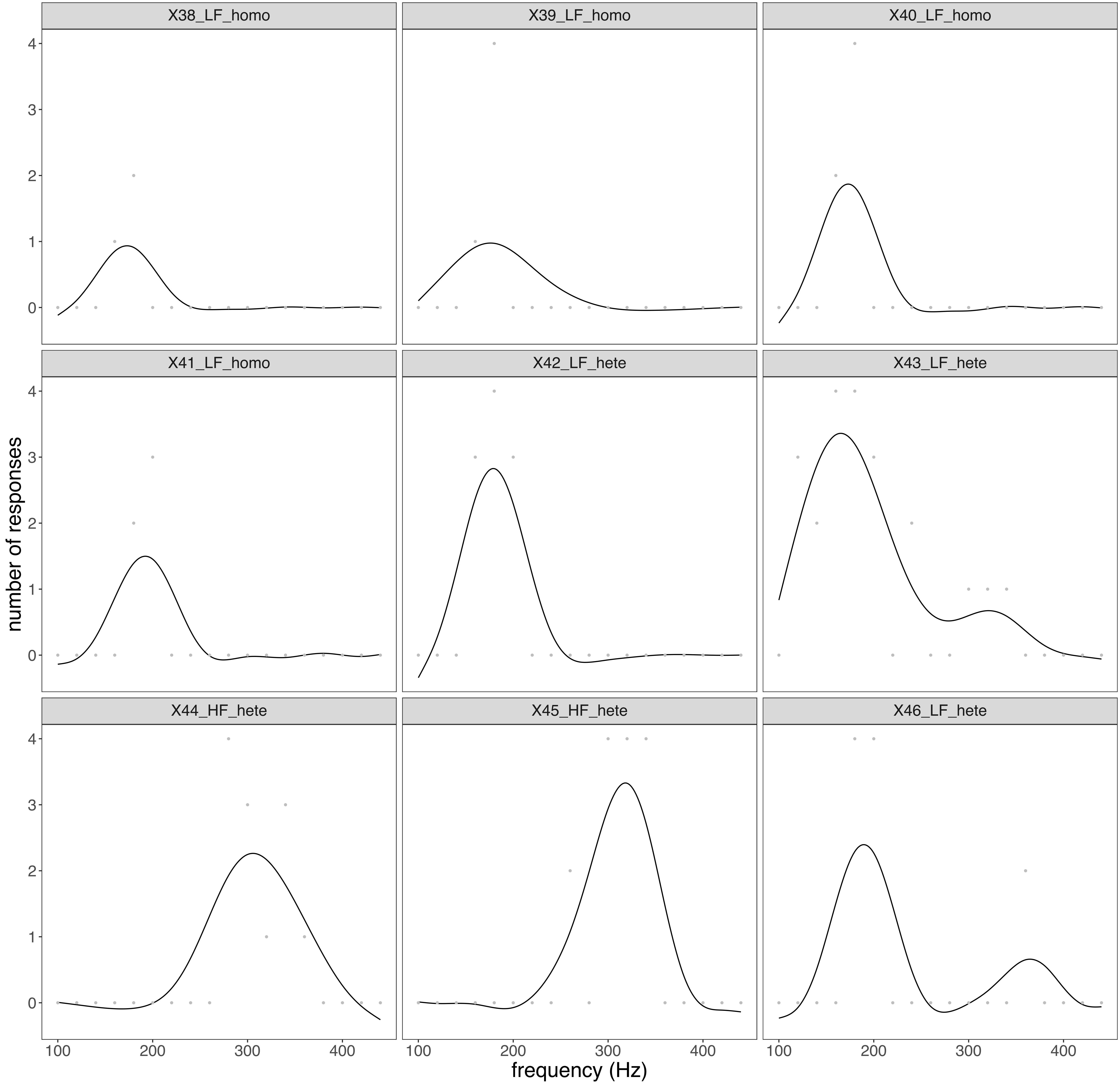
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frequency (Hz)
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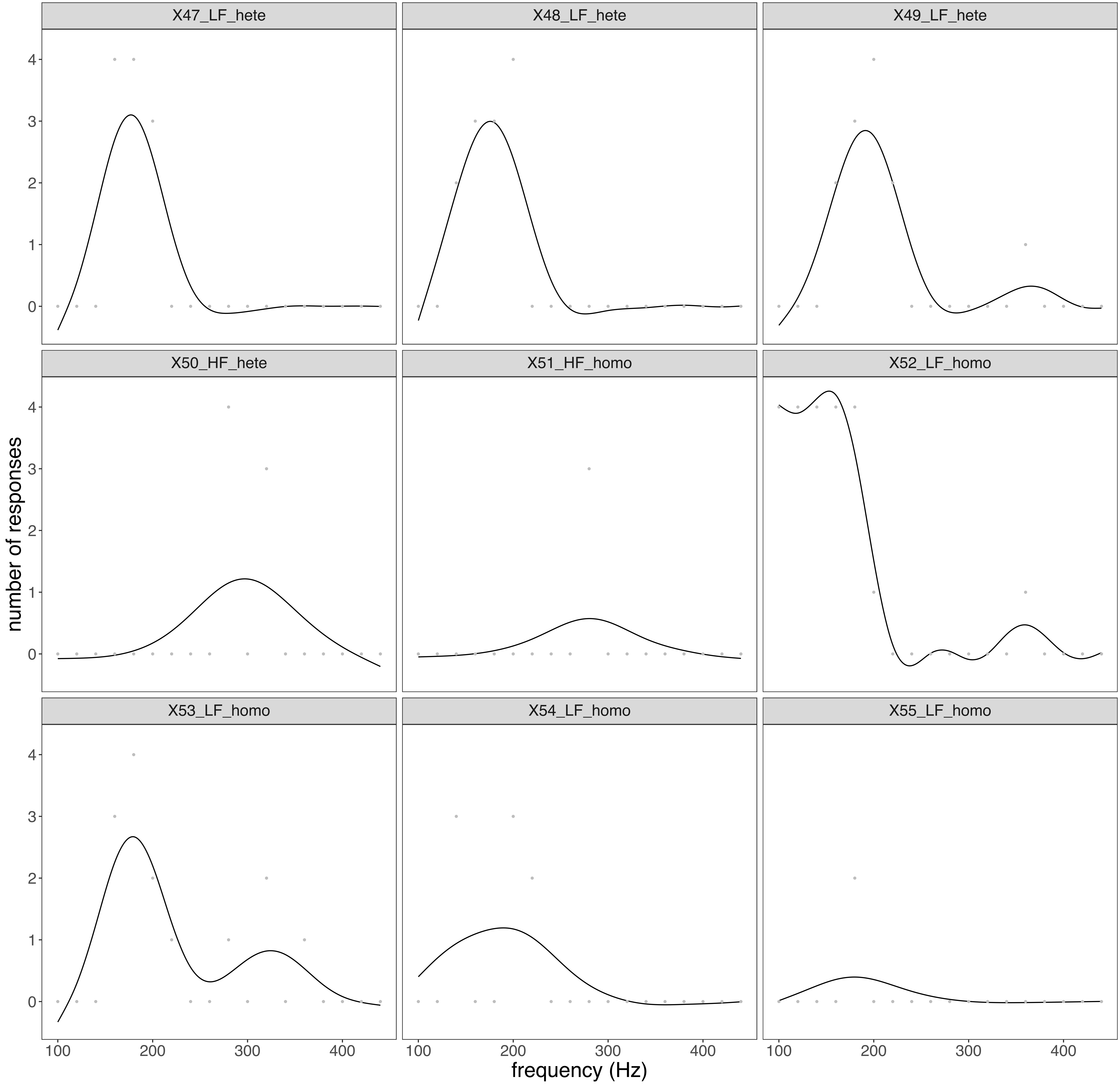
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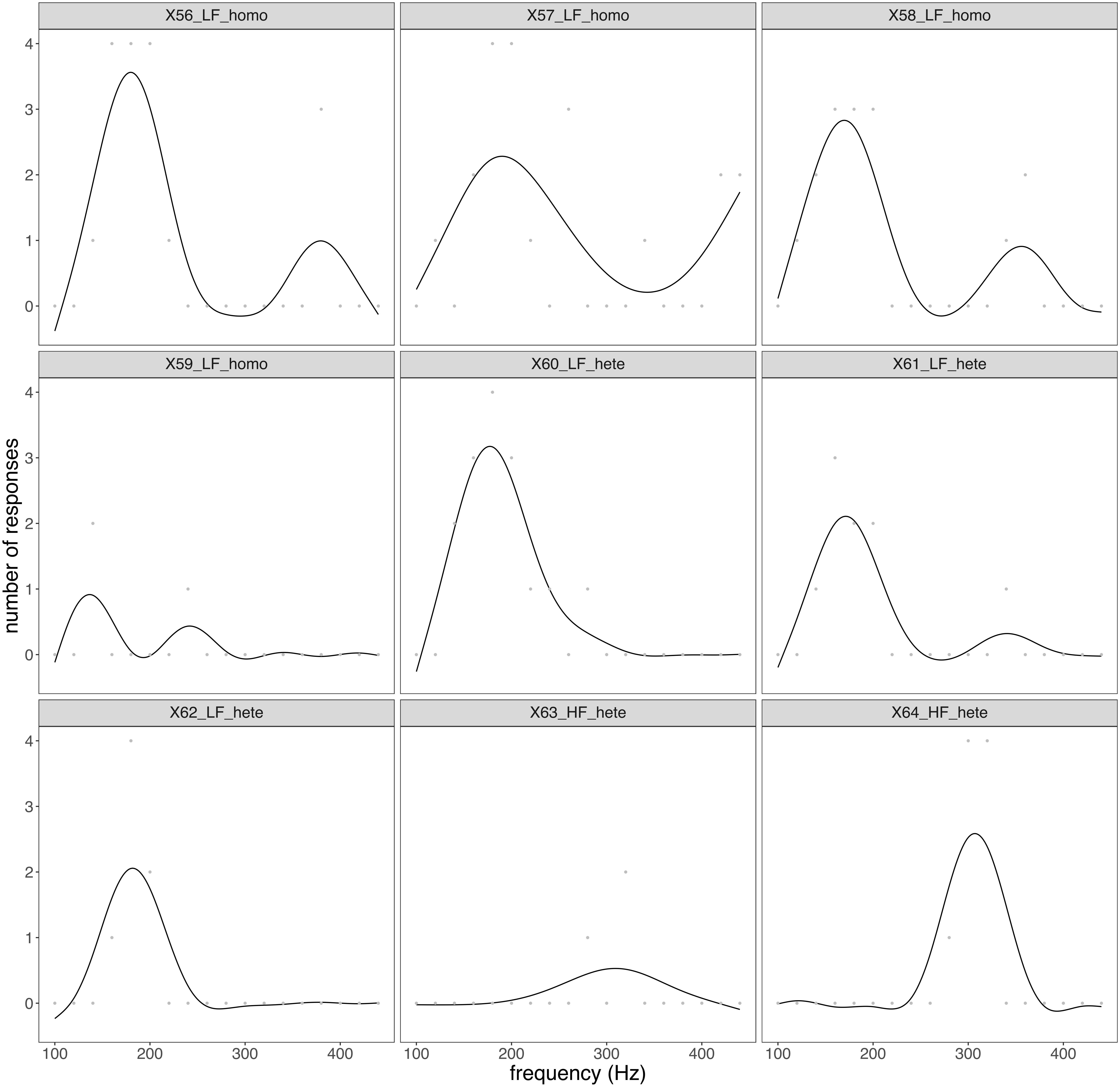
frequency (Hz)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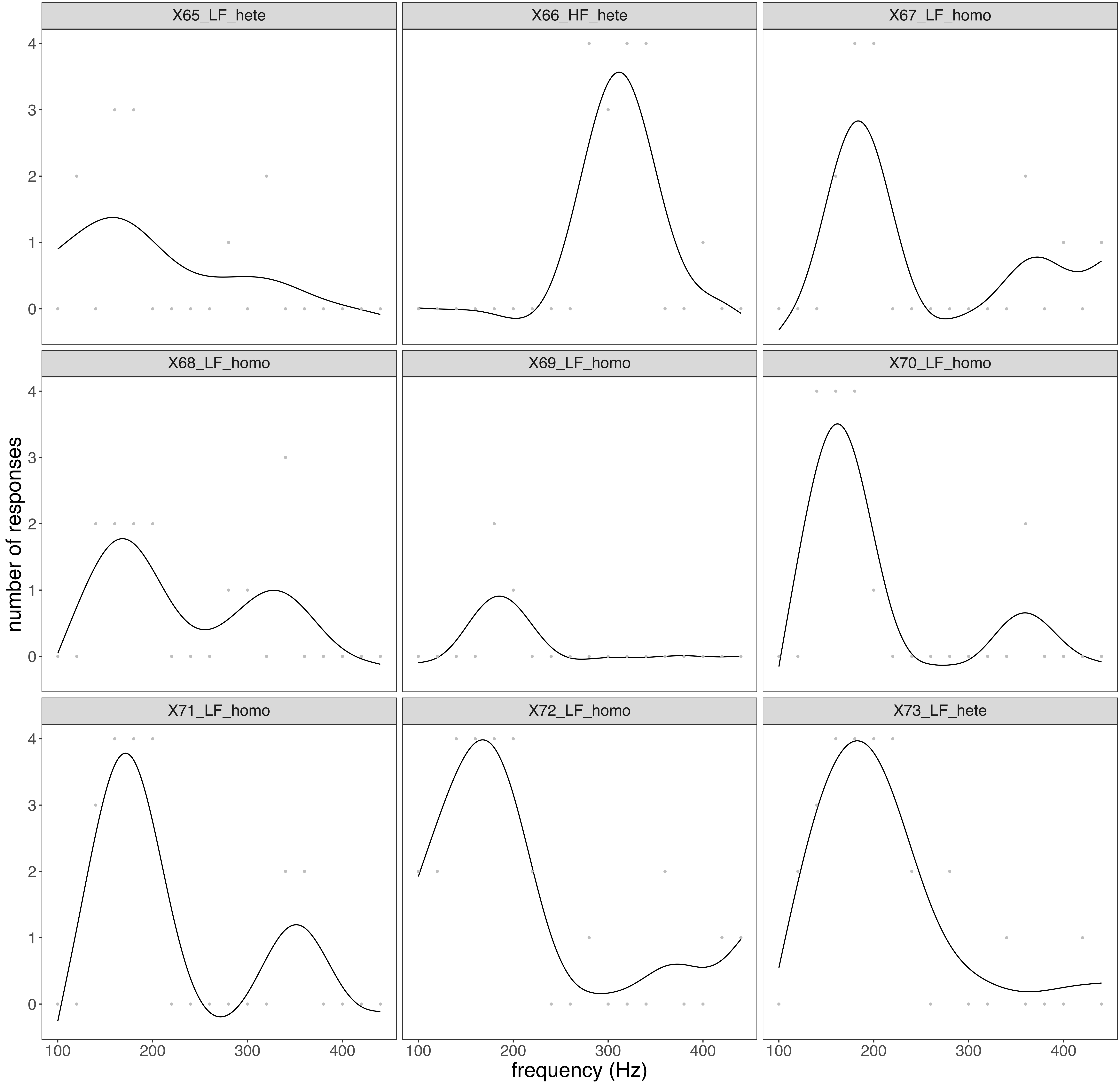
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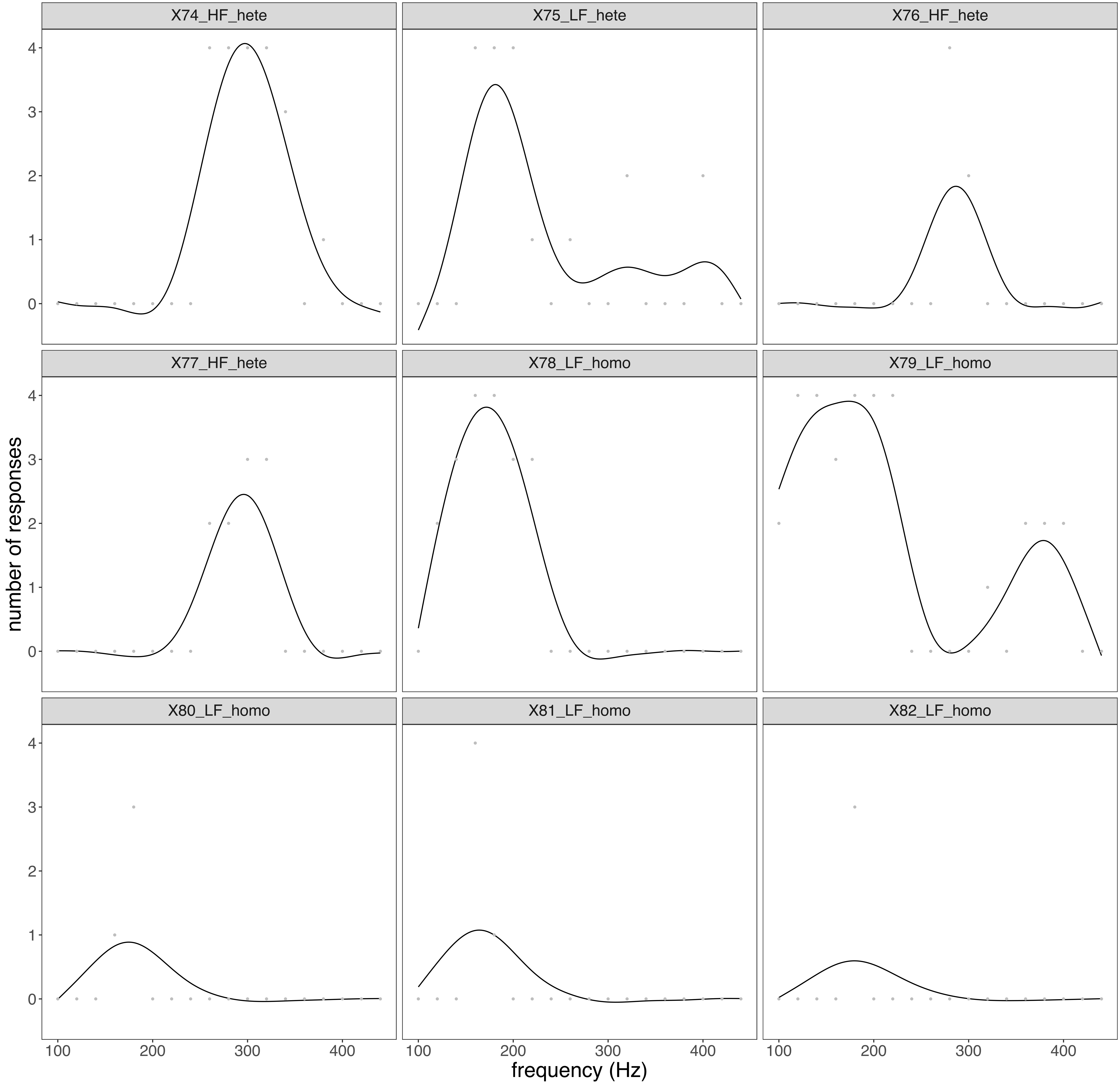
frequency (Hz)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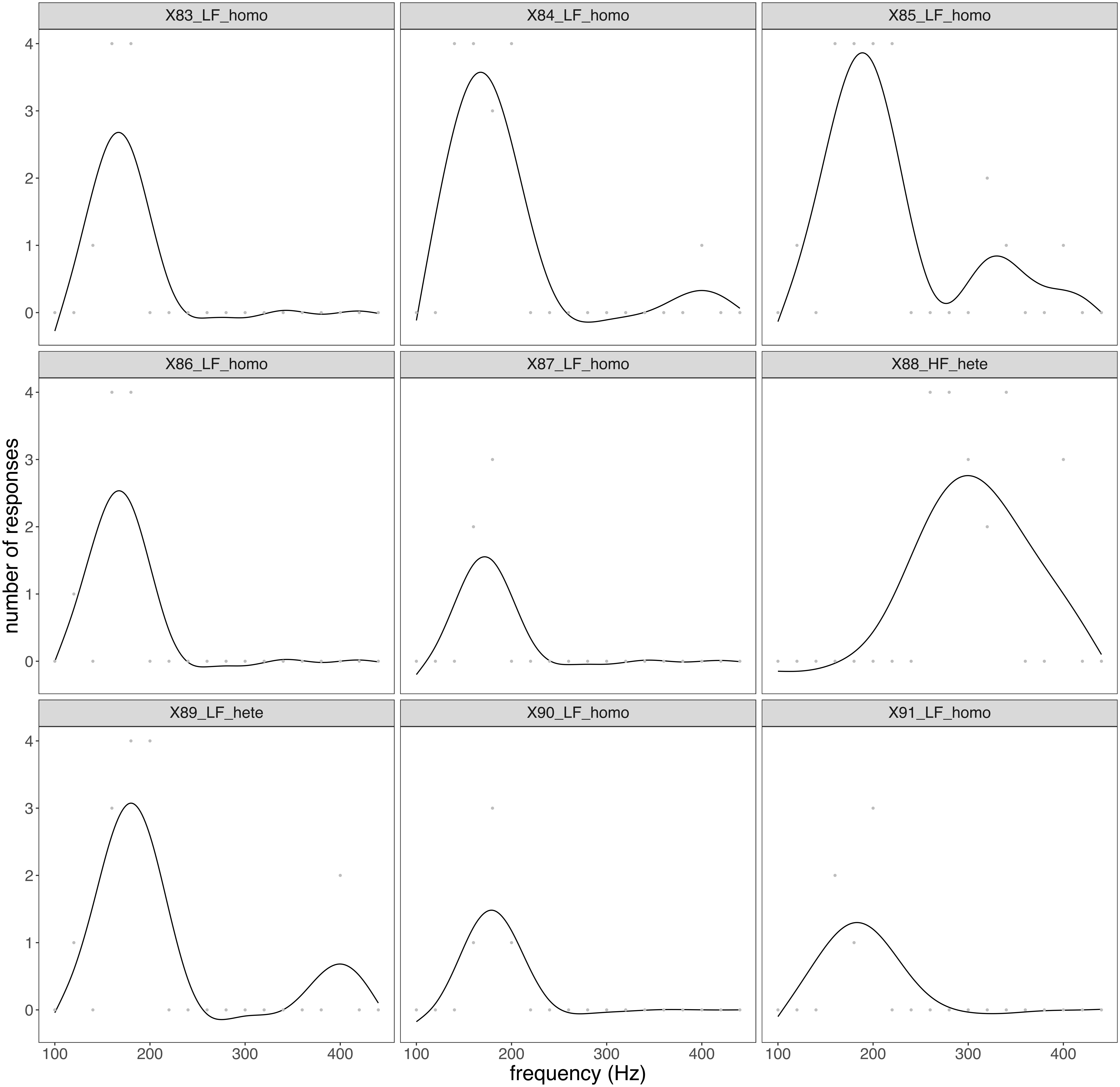
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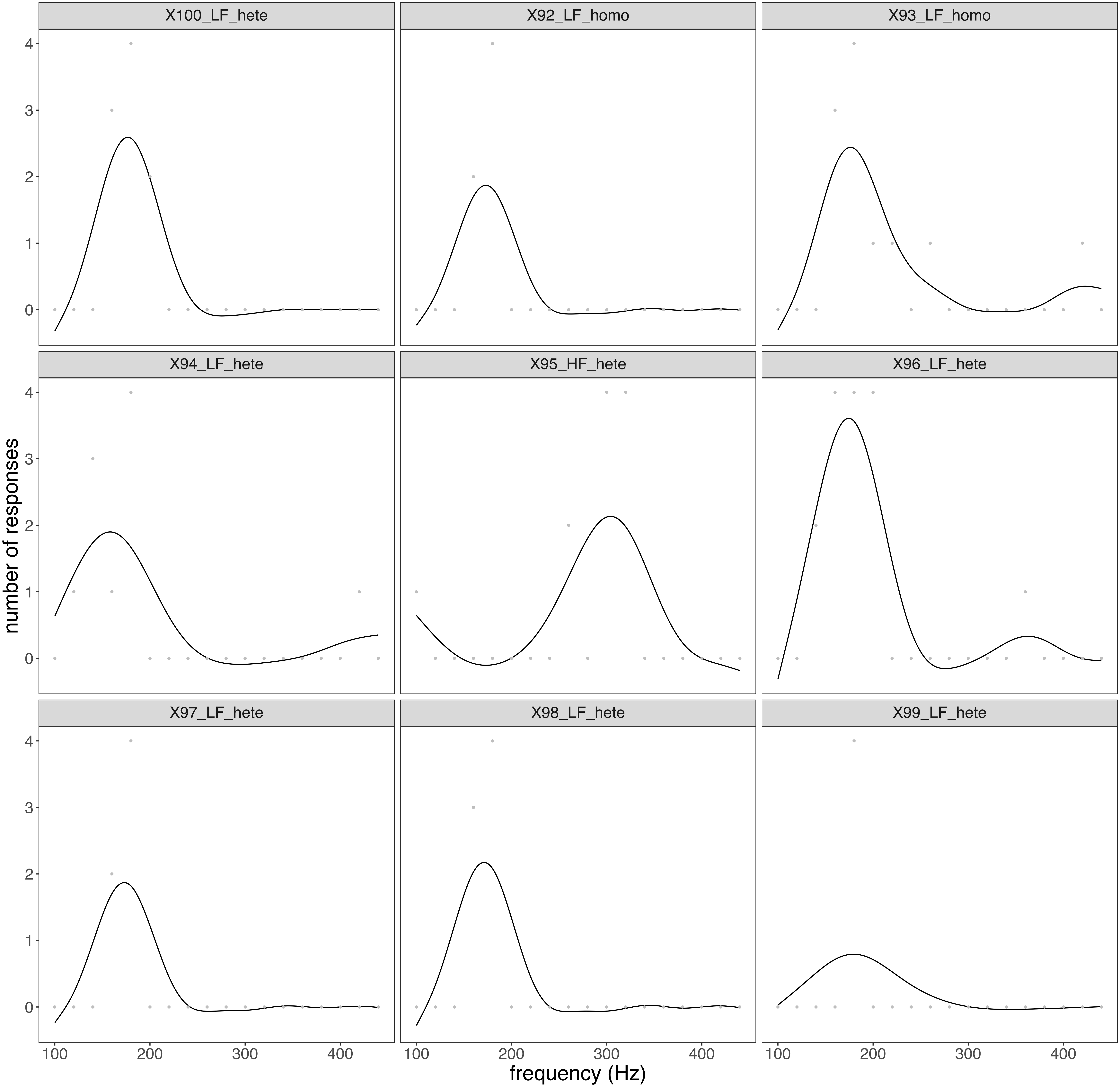
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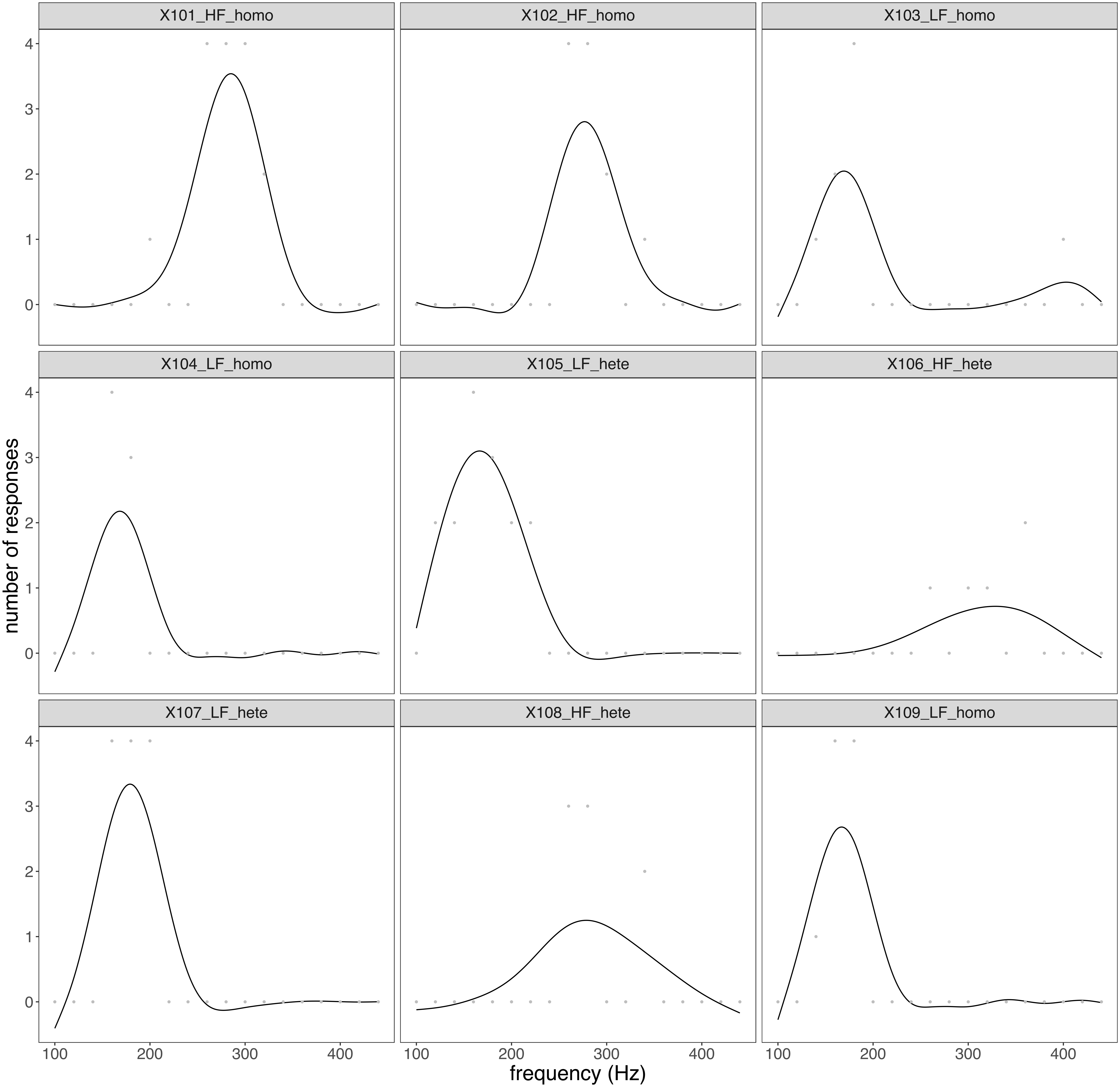
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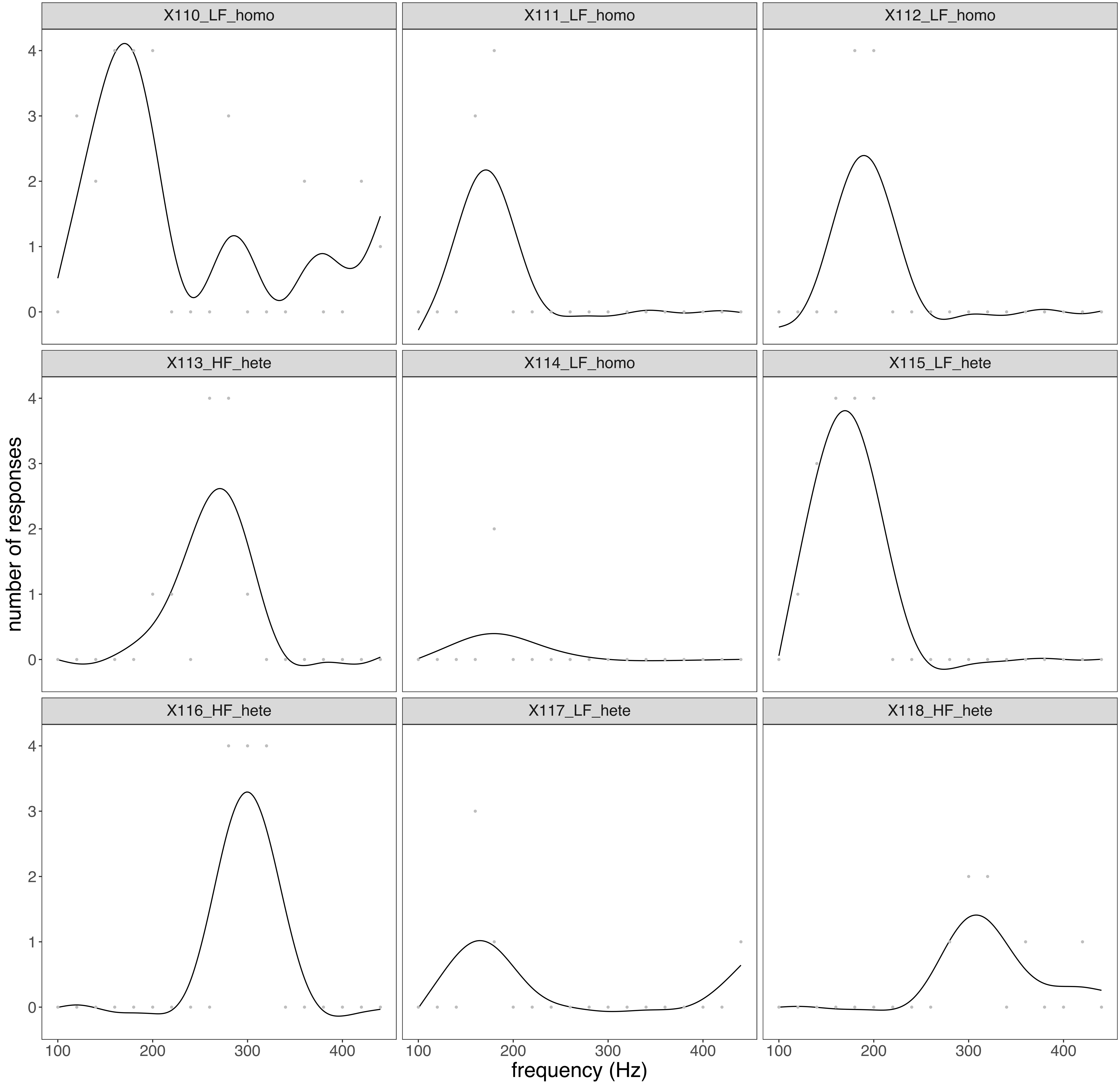
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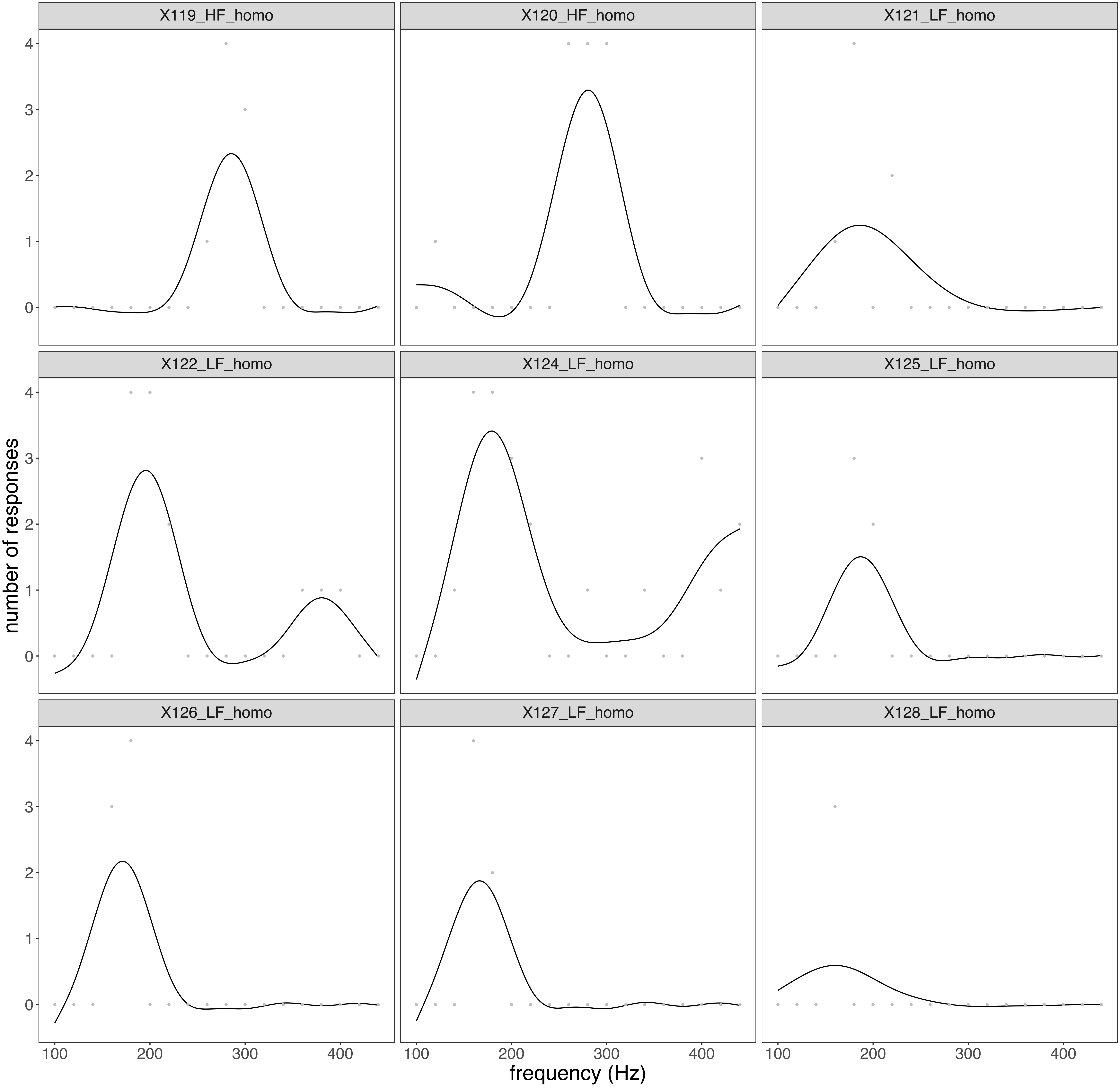
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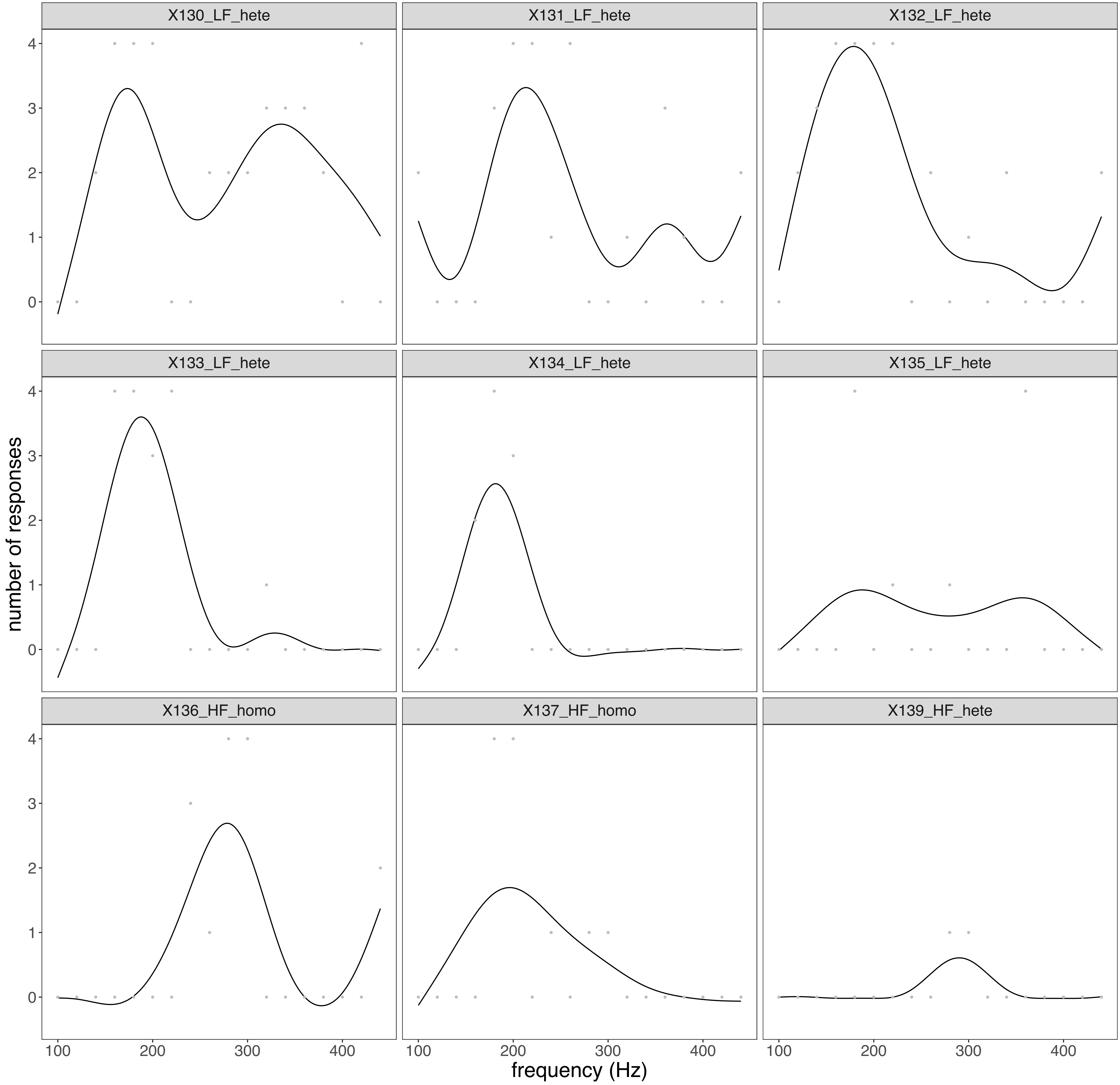
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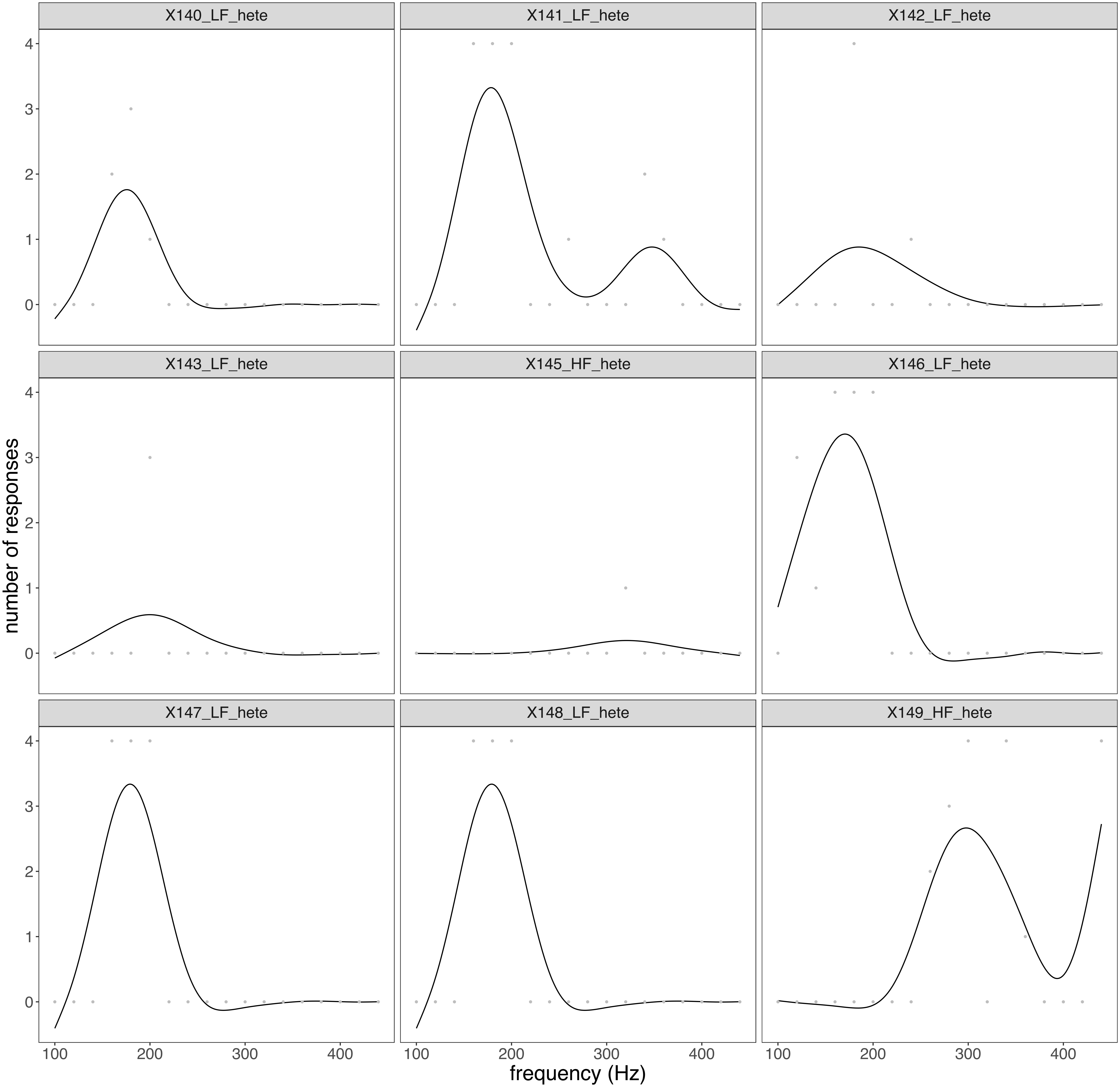
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frequency (Hz)
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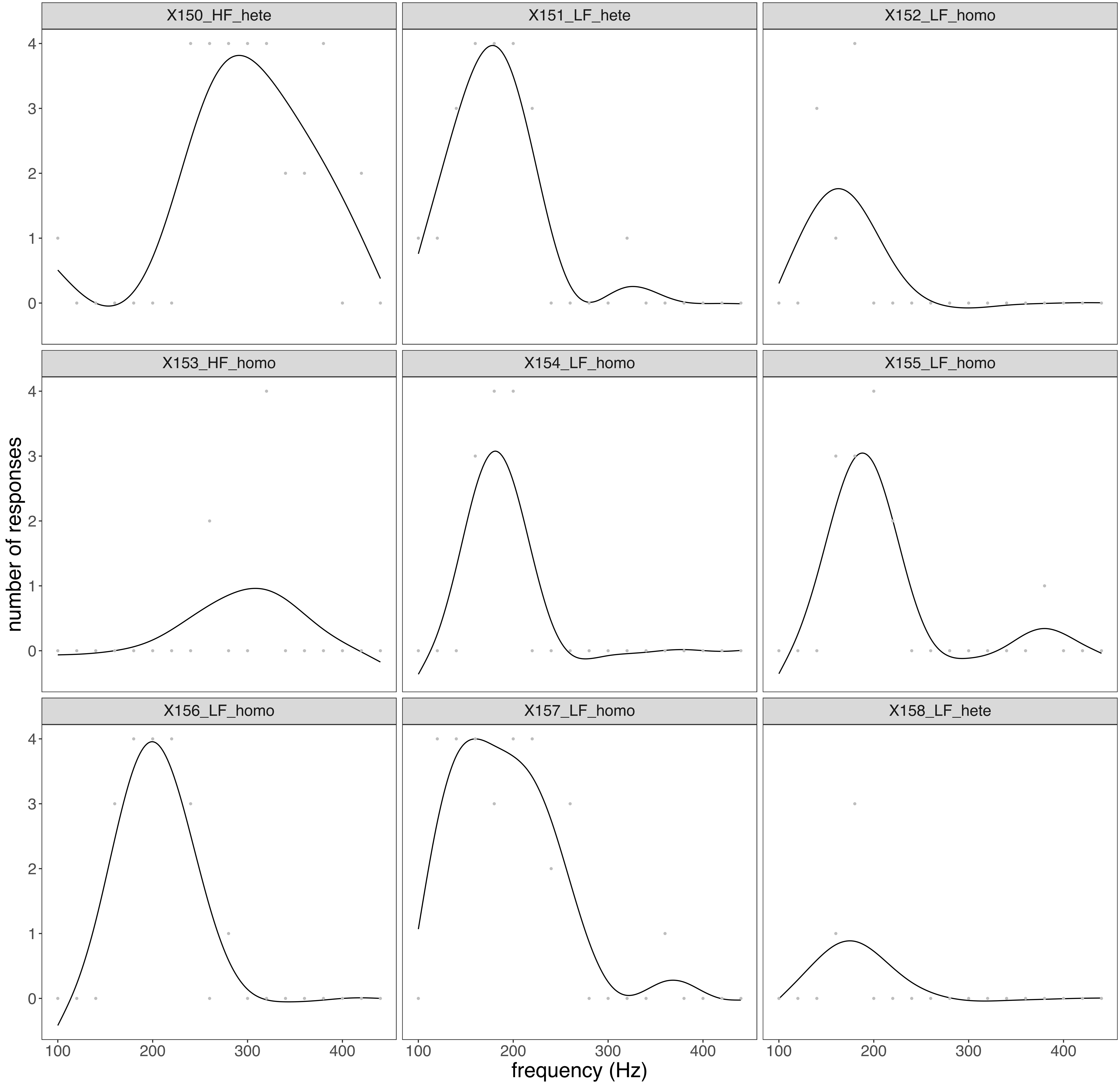
200
300
400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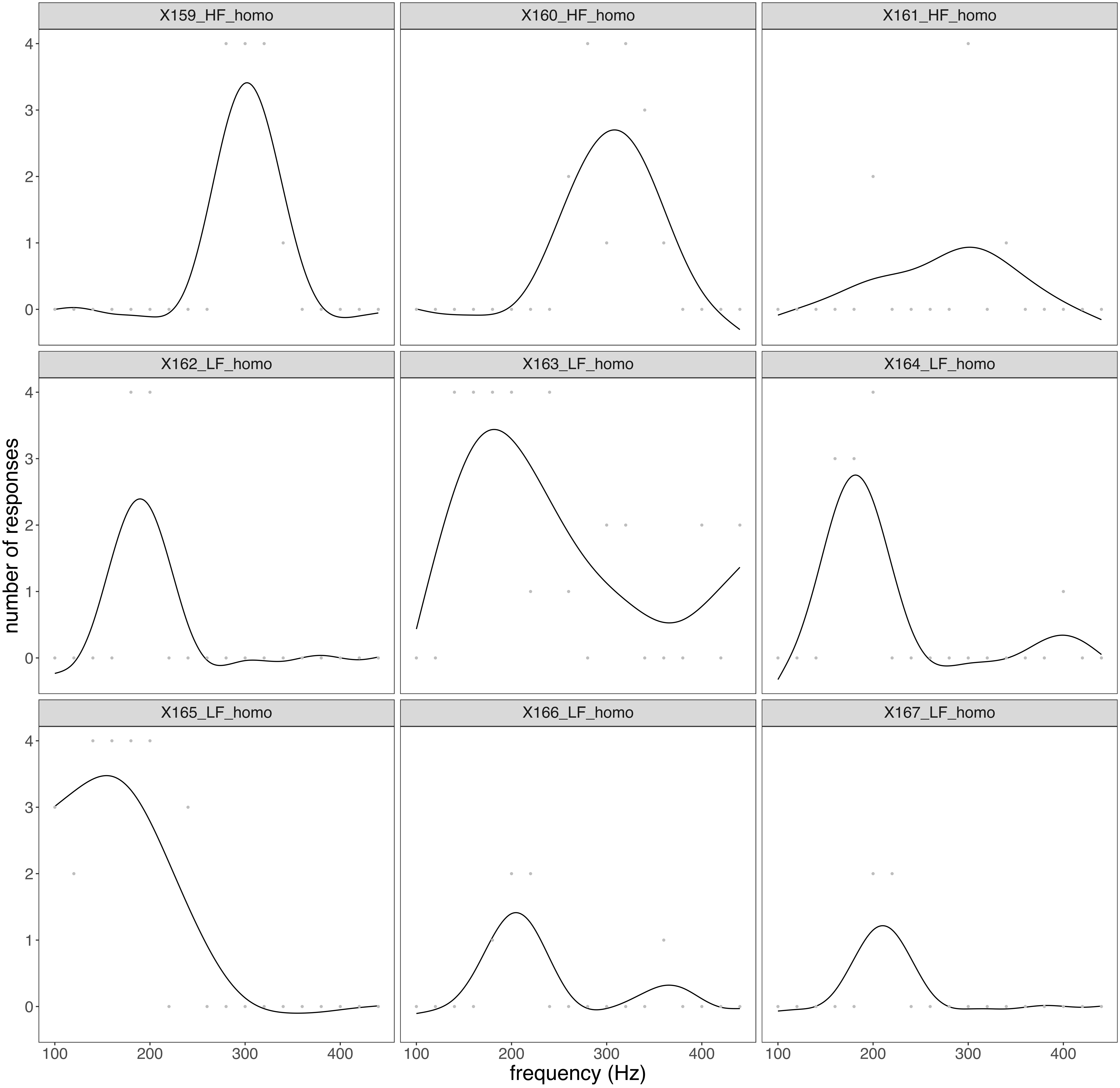
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300
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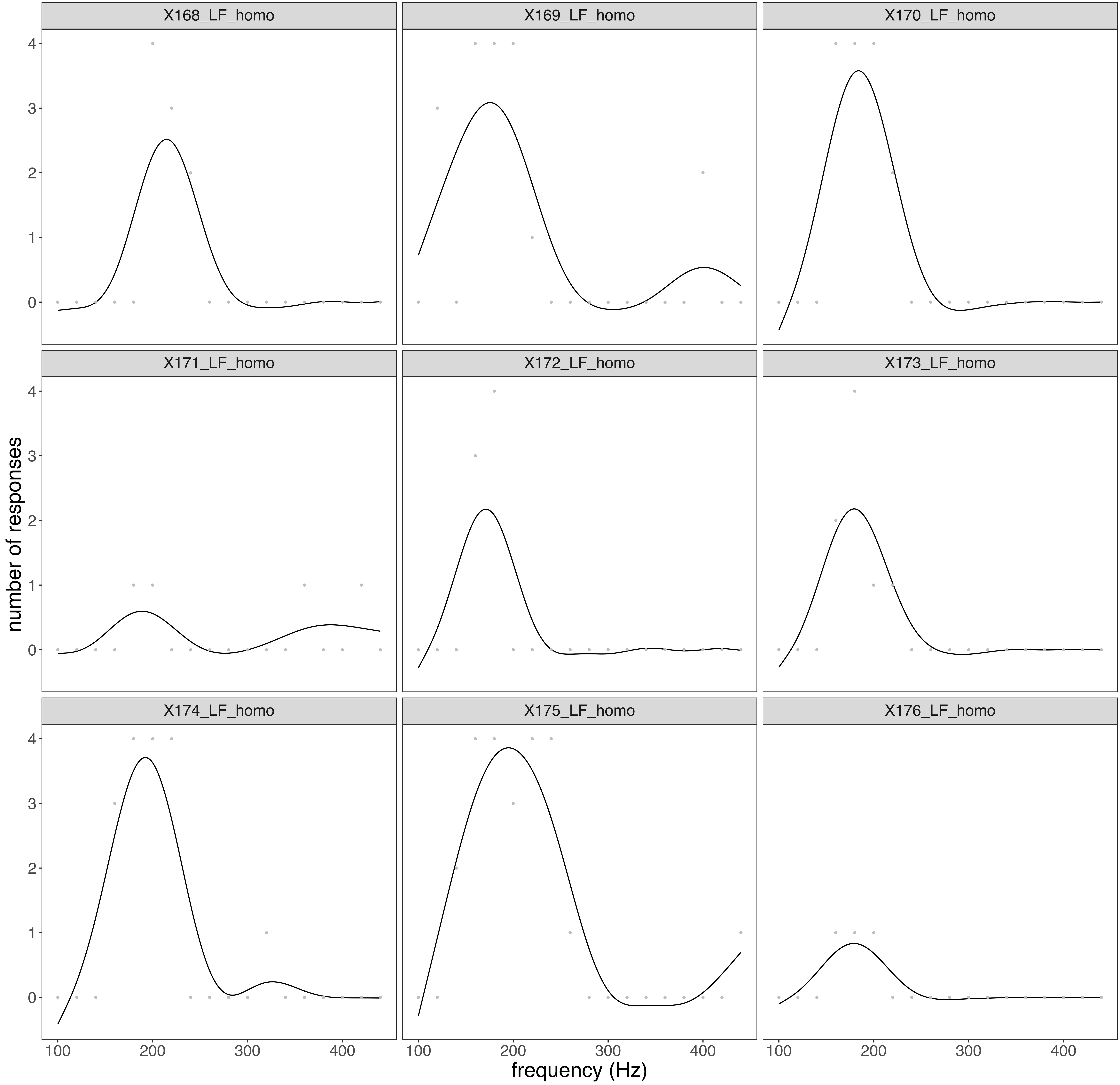
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200
300
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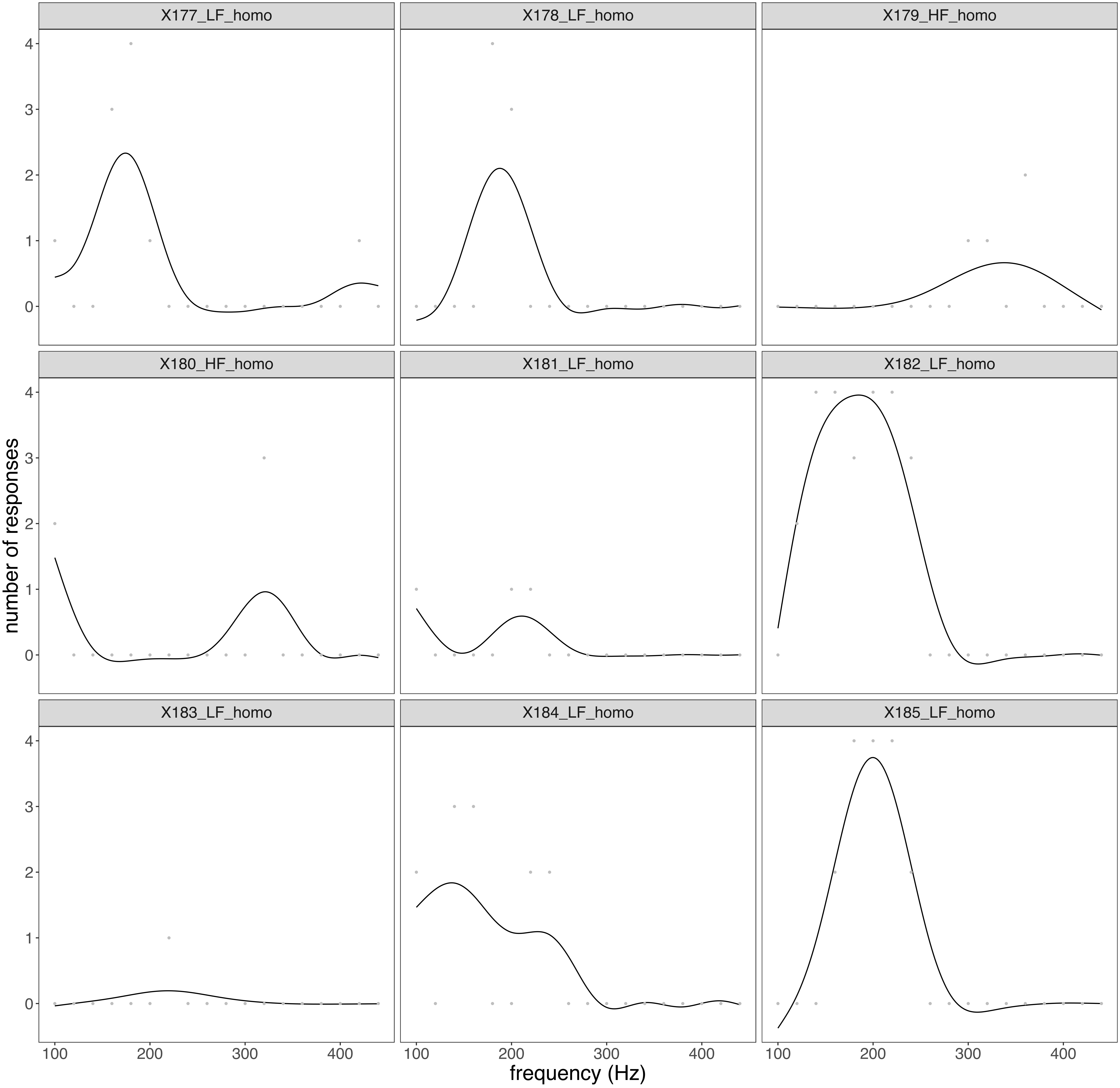
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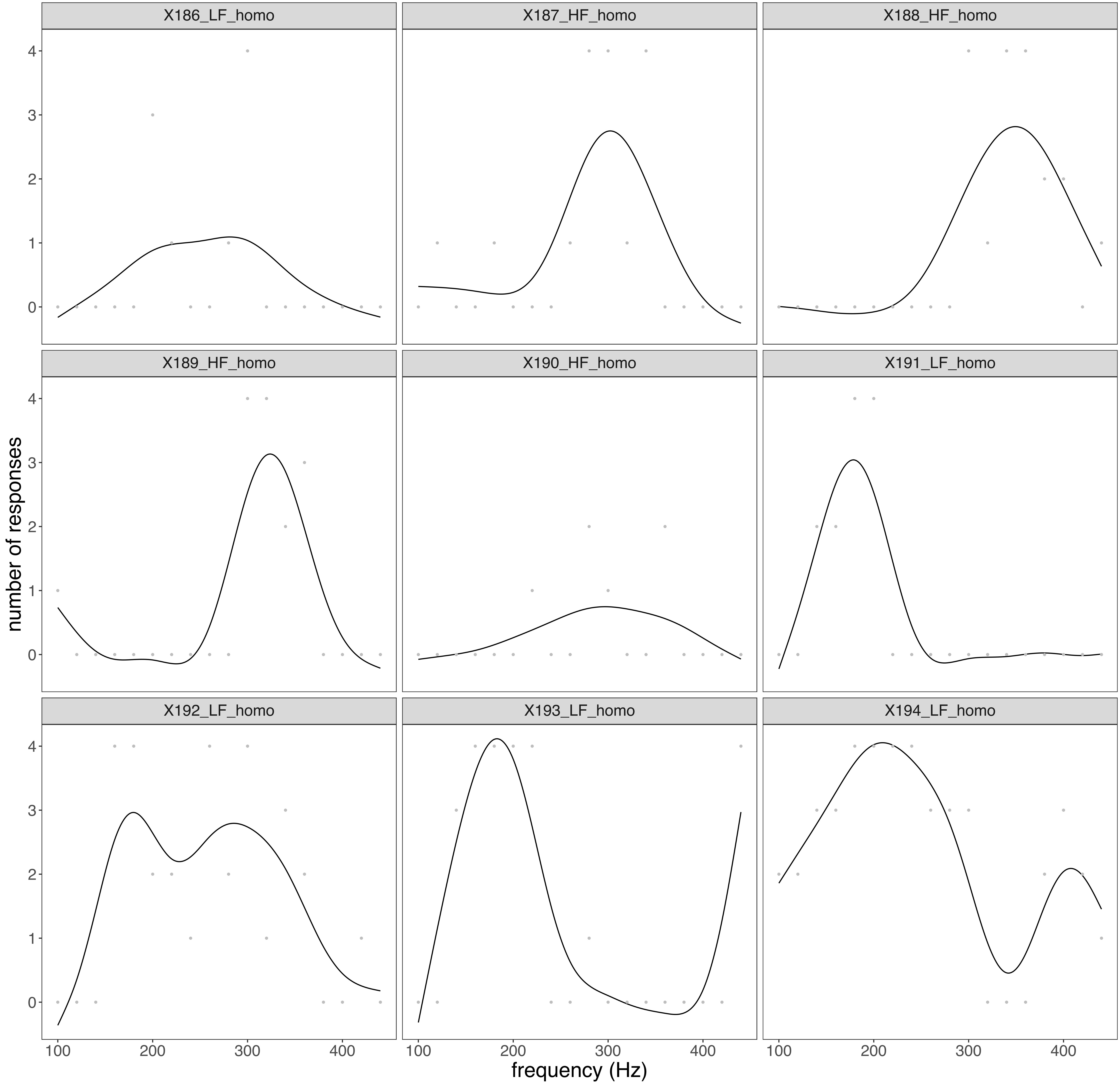
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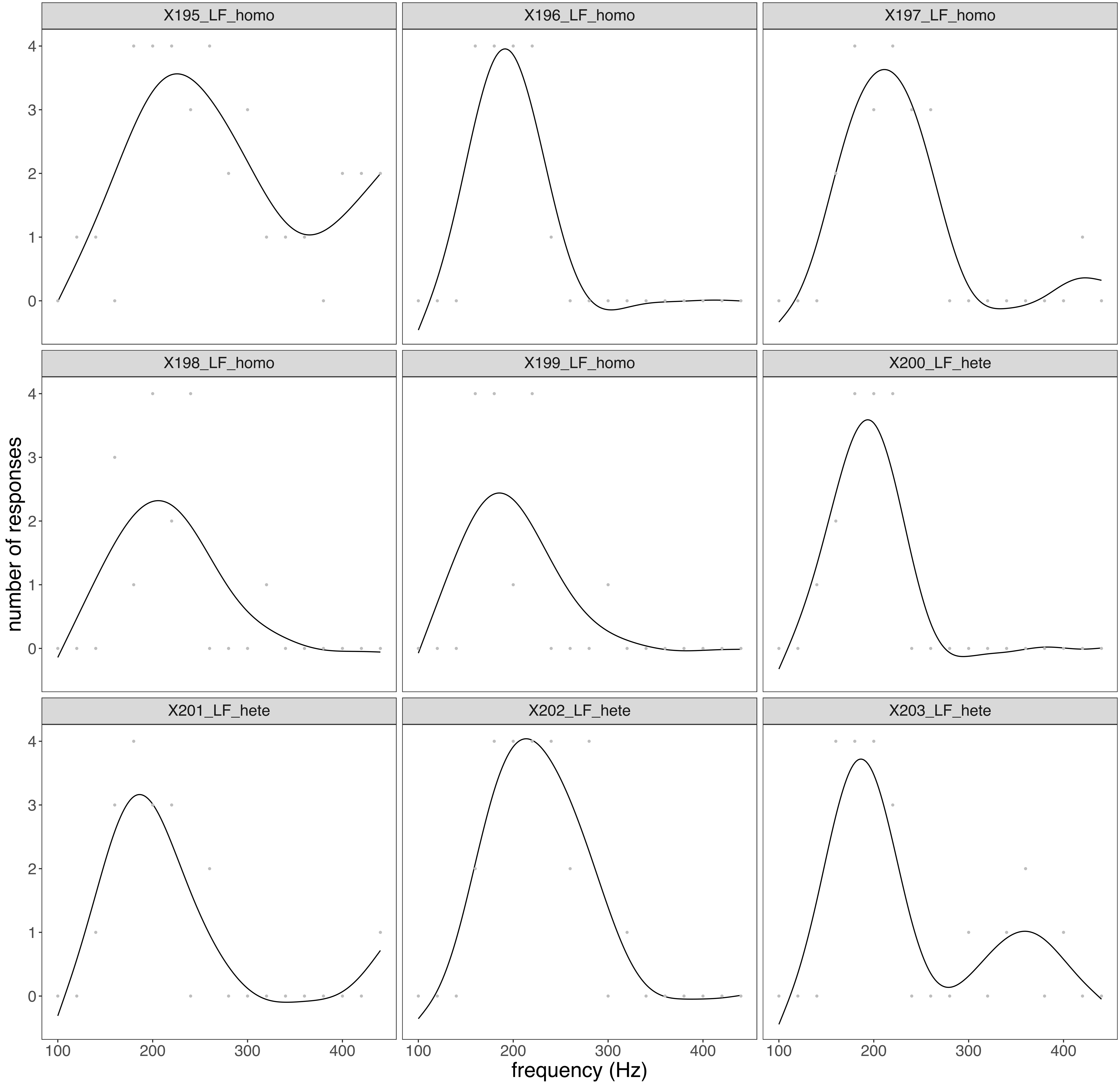
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frequency (Hz)
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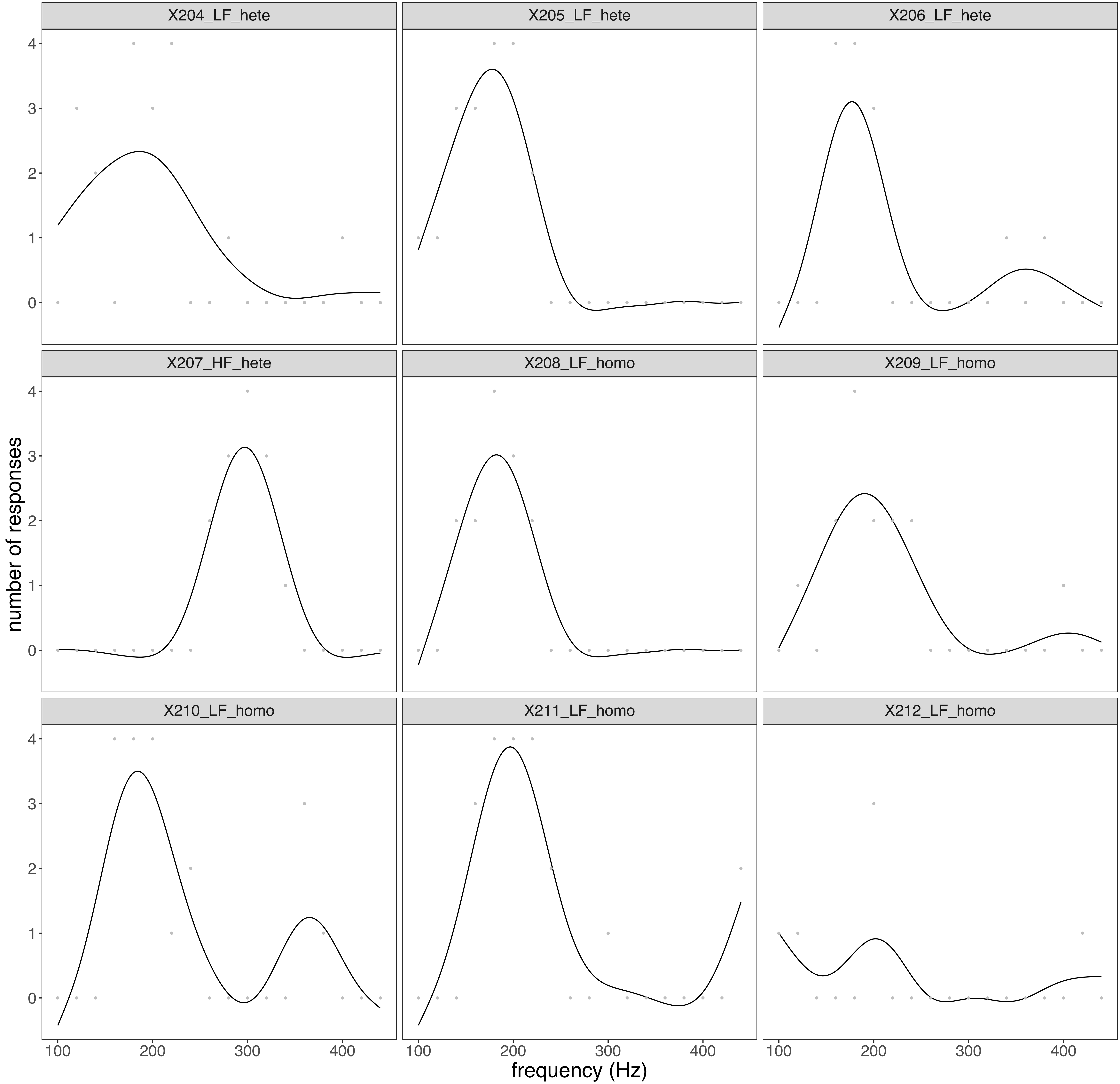
200
300
400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frequency (Hz)
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200
300
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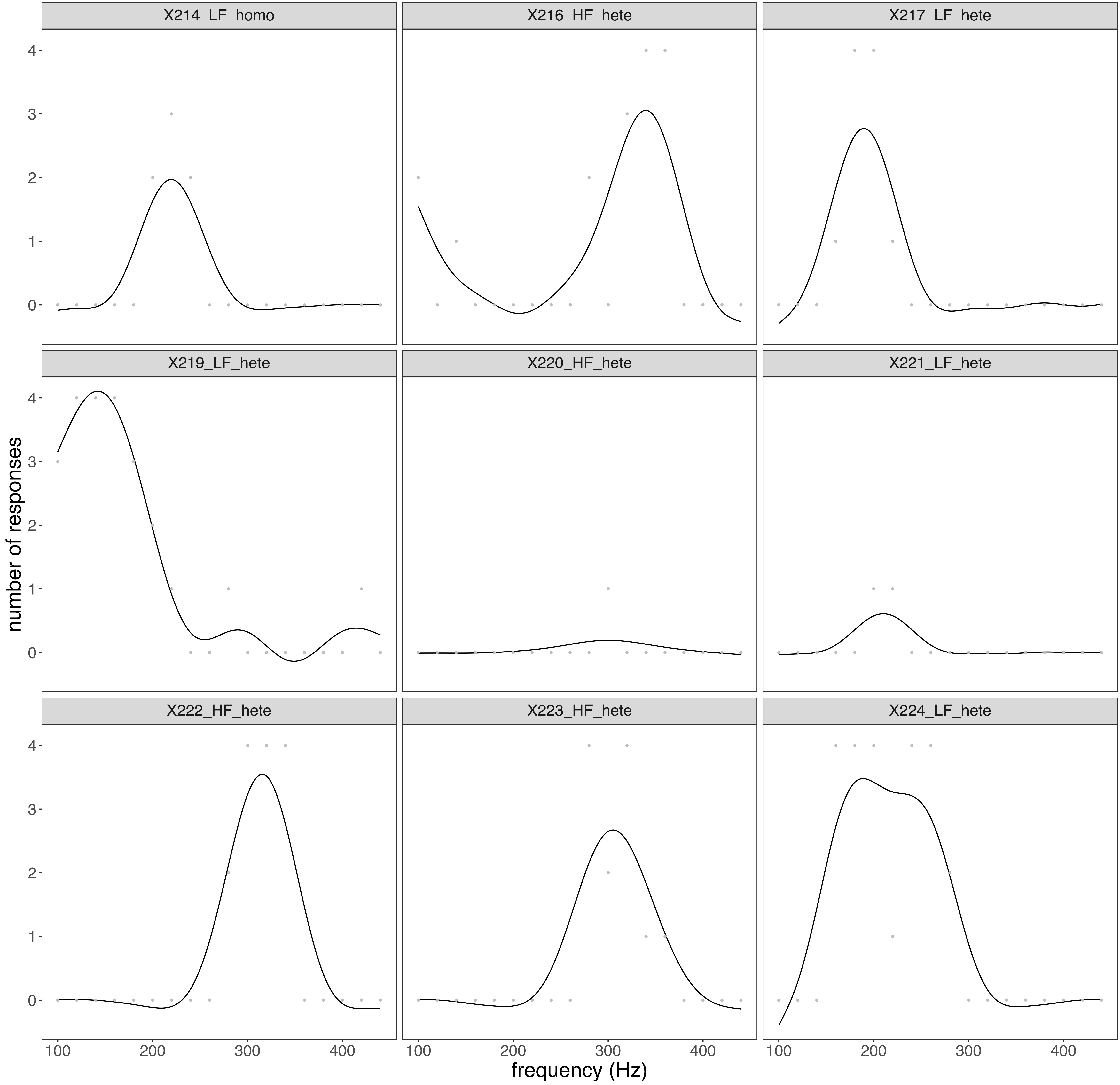
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frequency (Hz)
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200
300
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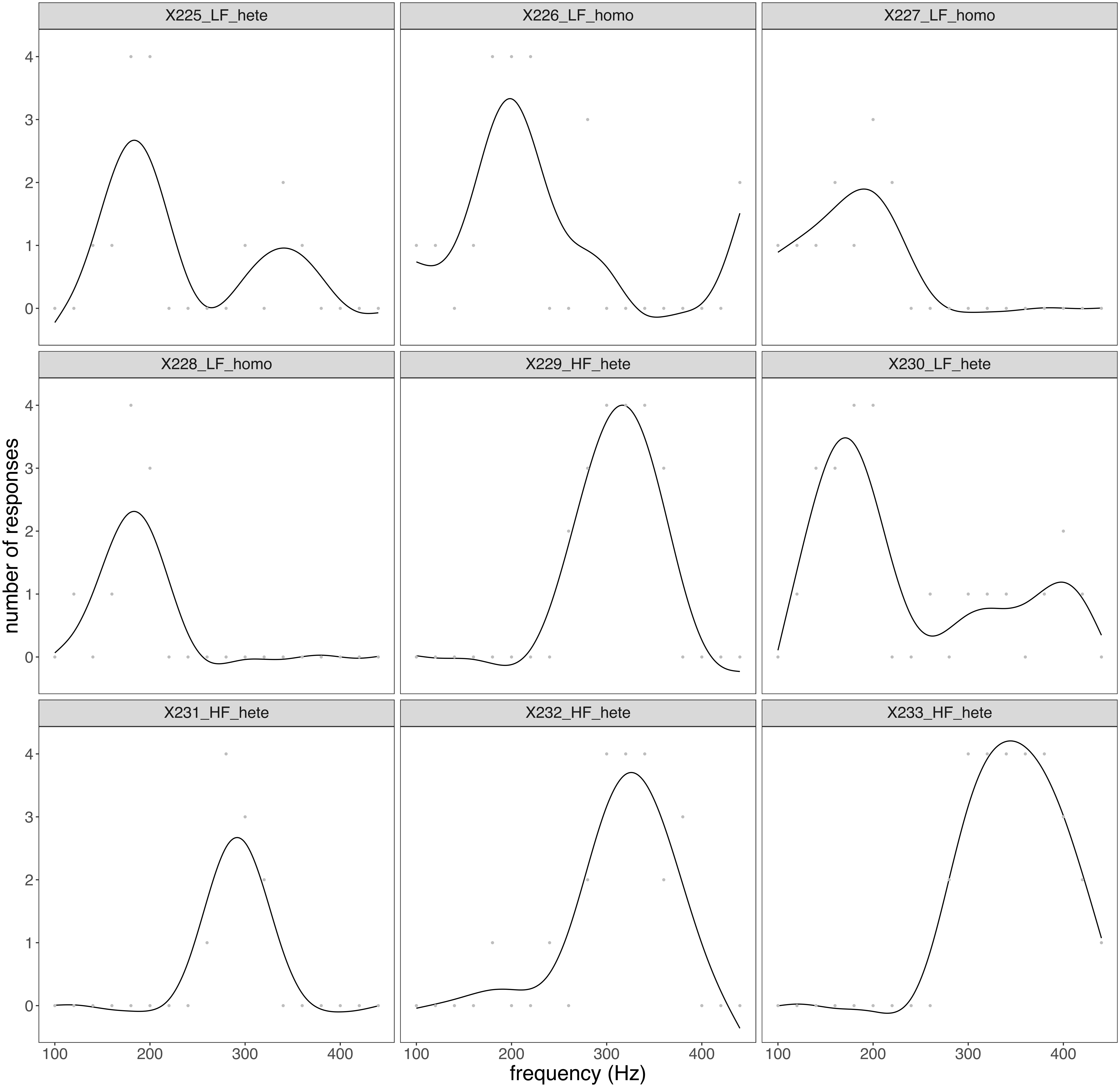
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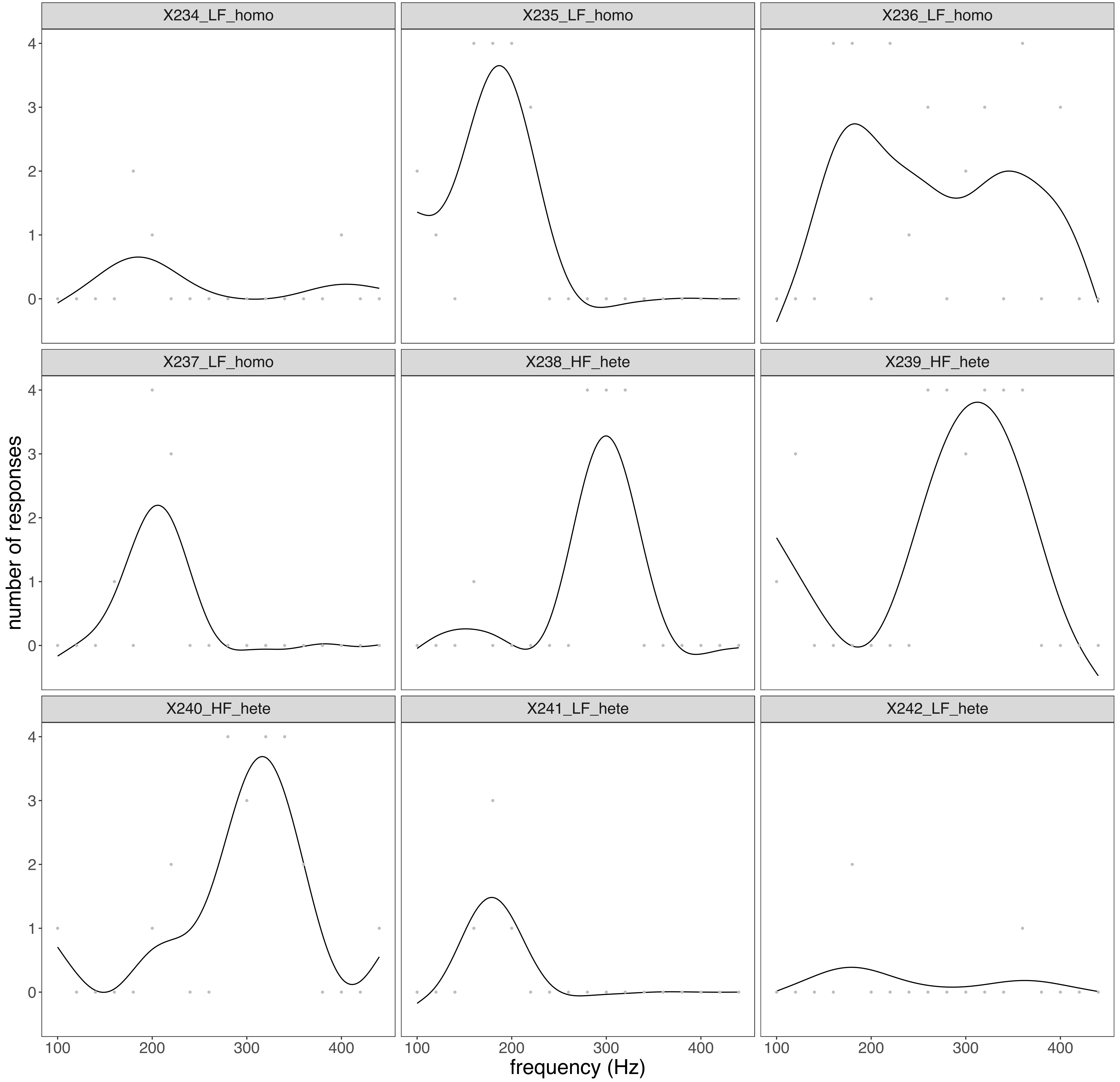
3
4
frequency (Hz)
100
200
300
400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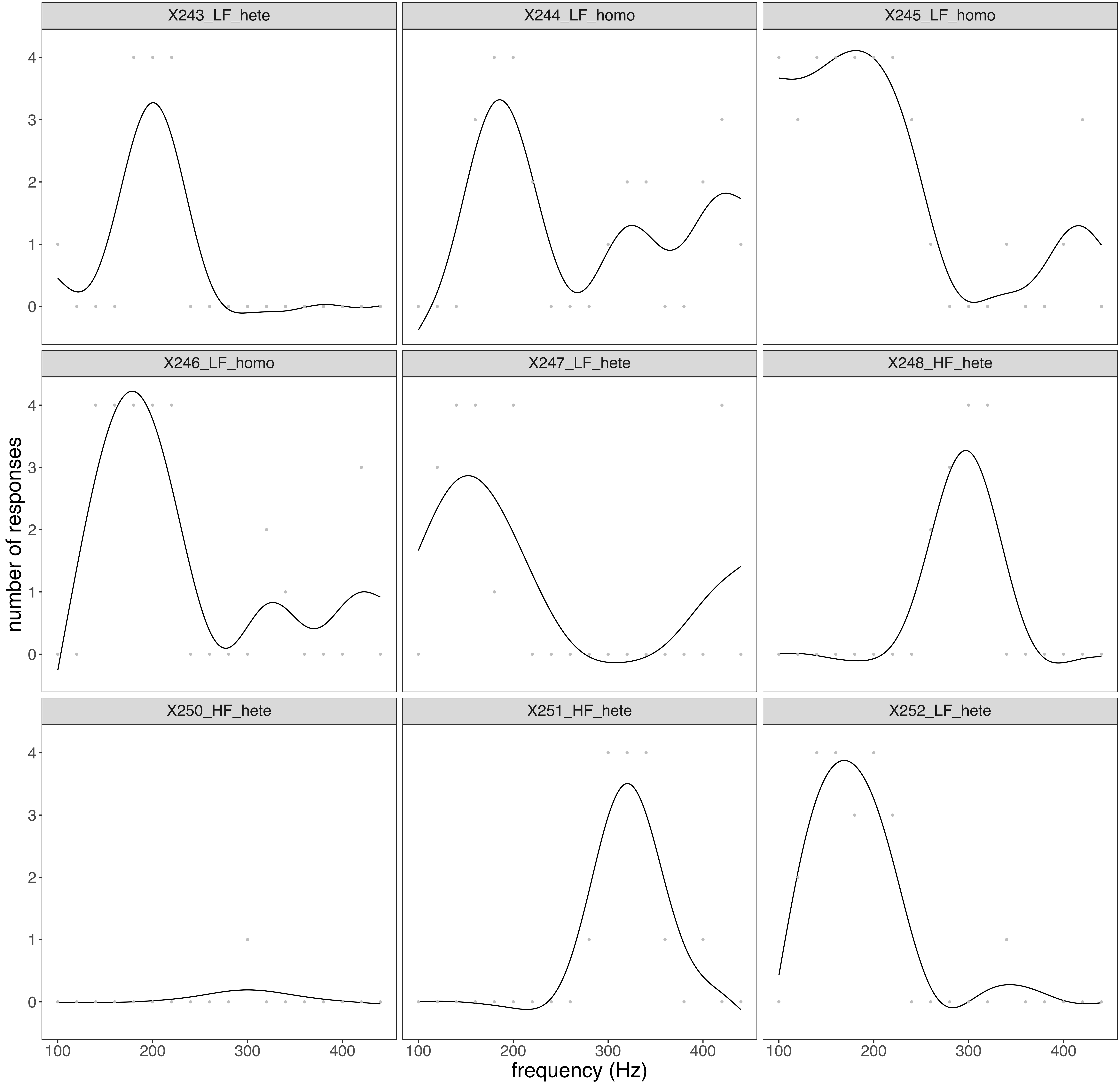
X238_HF_hete
X239_HF_hete
X240_HF_hete
X241_LF_hete
X242_LF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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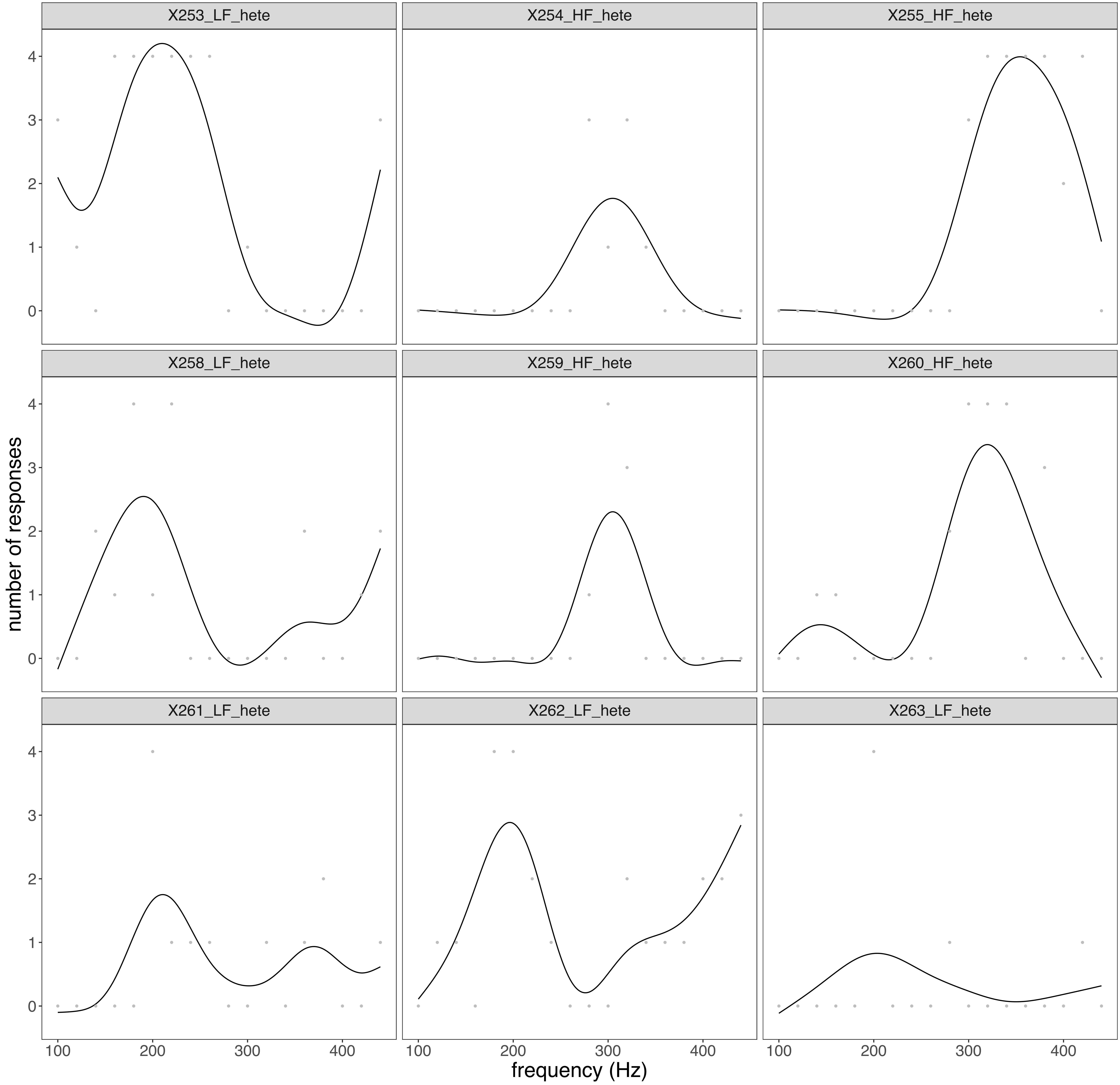
X245_LF_homo
X246_LF_homo
X247_LF_hete
X248_HF_hete
X250_HF_hete
X251_HF_hete
X252_LF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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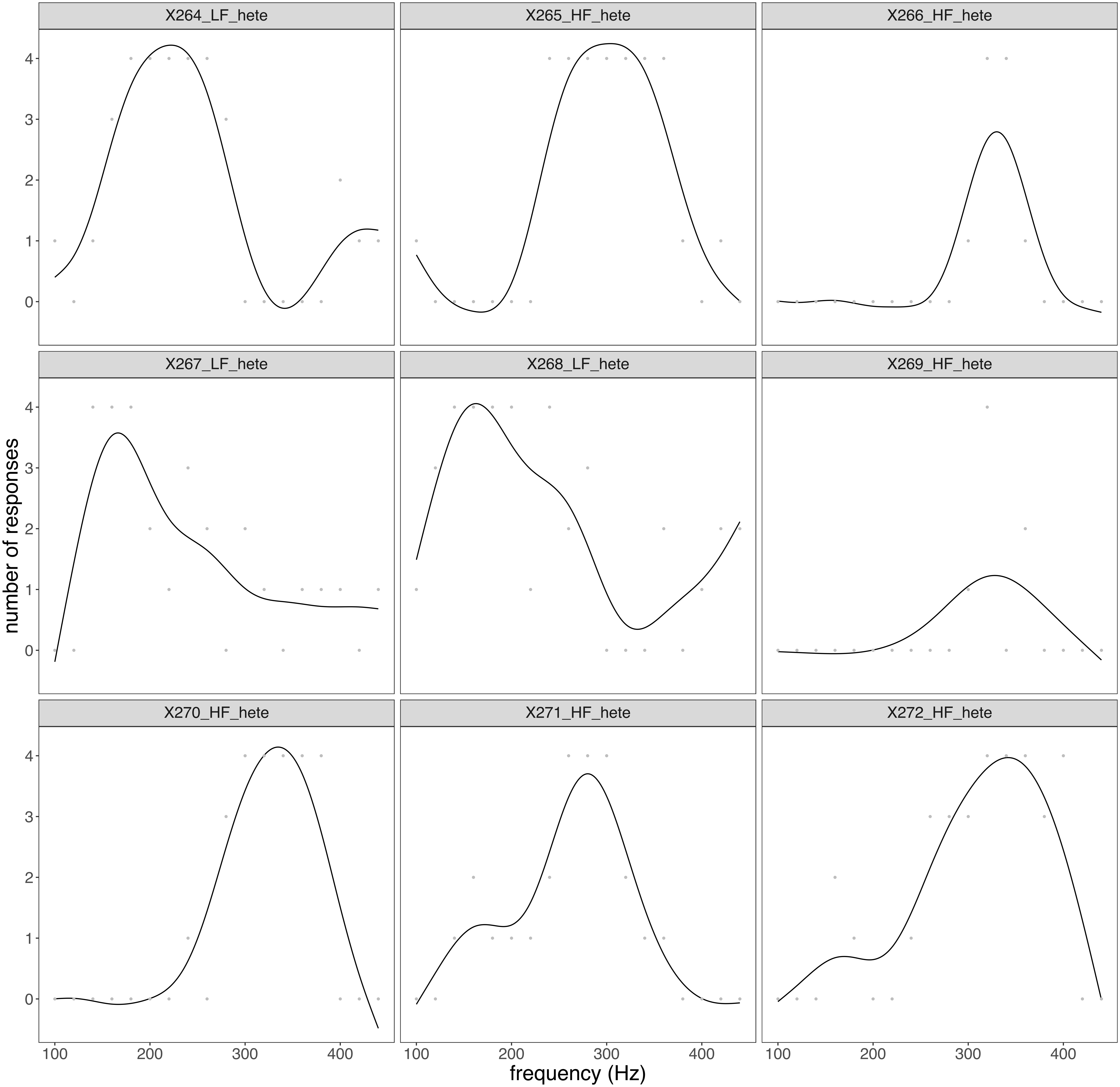
X255_HF_hete
X258_LF_hete
X259_HF_hete
X260_HF_hete
X261_LF_hete
X262_LF_hete
X263_LF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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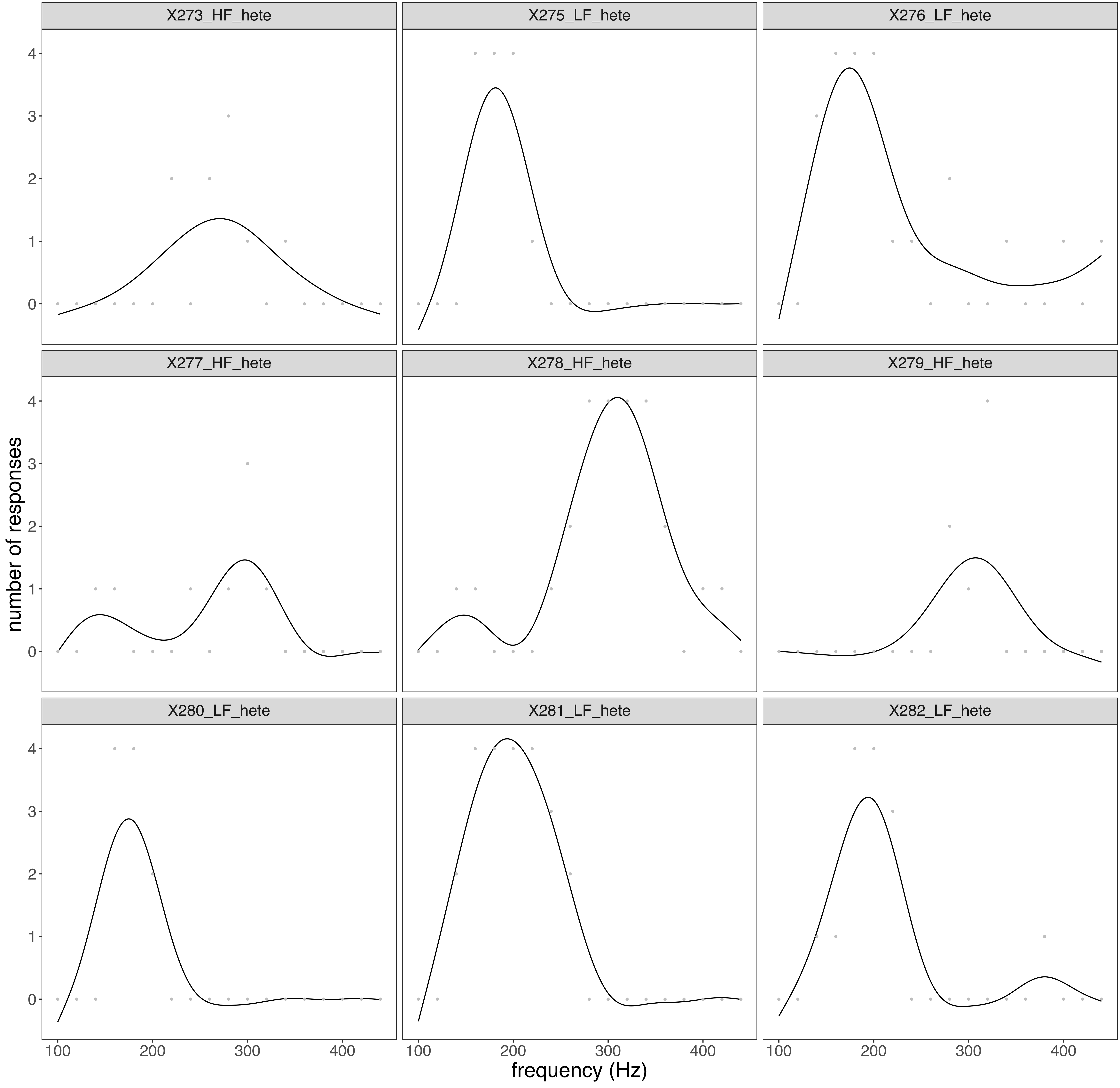
X266_HF_hete
X267_LF_hete
X268_LF_hete
X269_HF_hete
X270_HF_hete
X271_HF_hete
X272_HF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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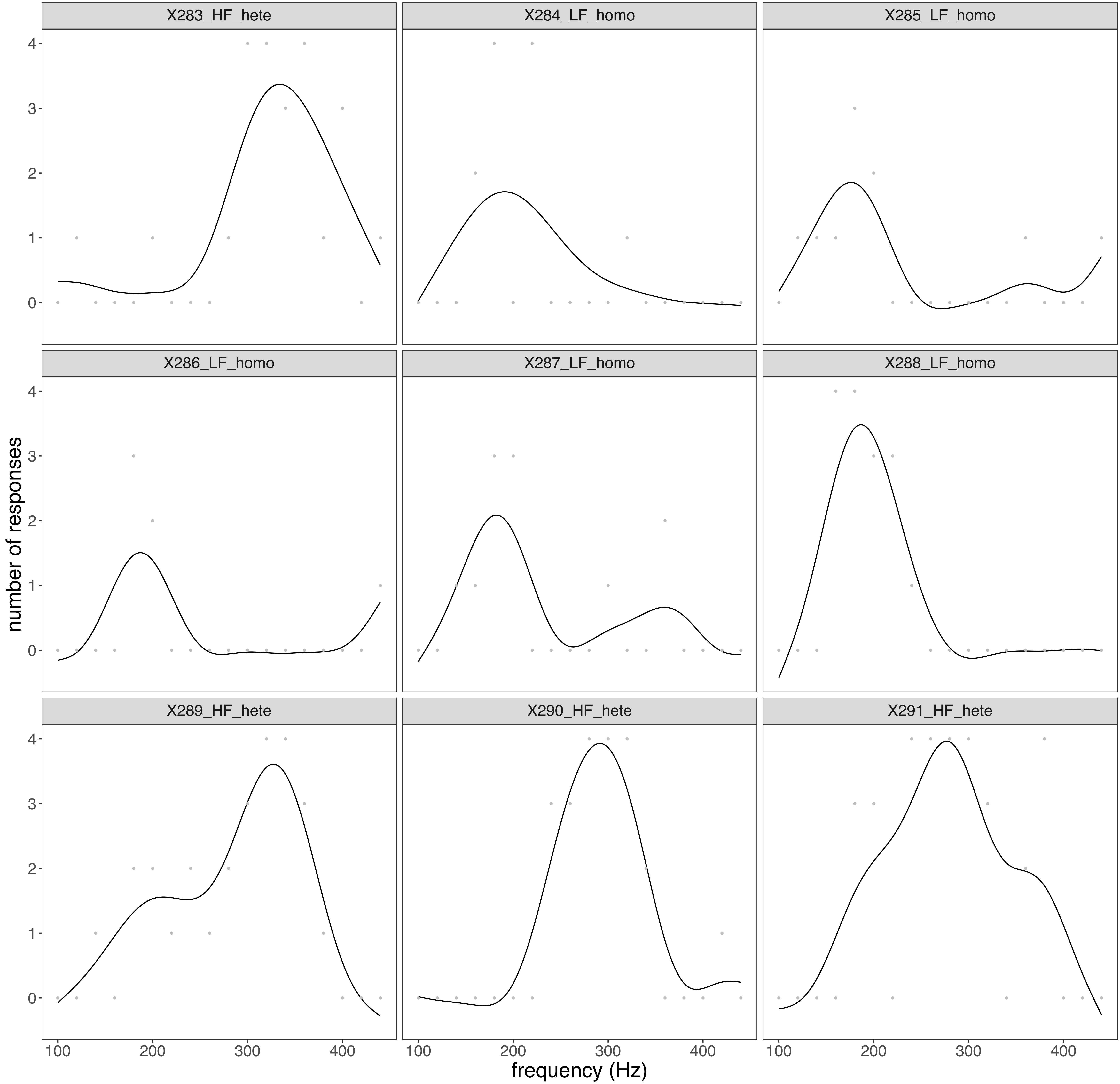
X276_LF_hete
X277_HF_hete
X278_HF_hete
X279_HF_hete
X280_LF_hete
X281_LF_hete
X282_LF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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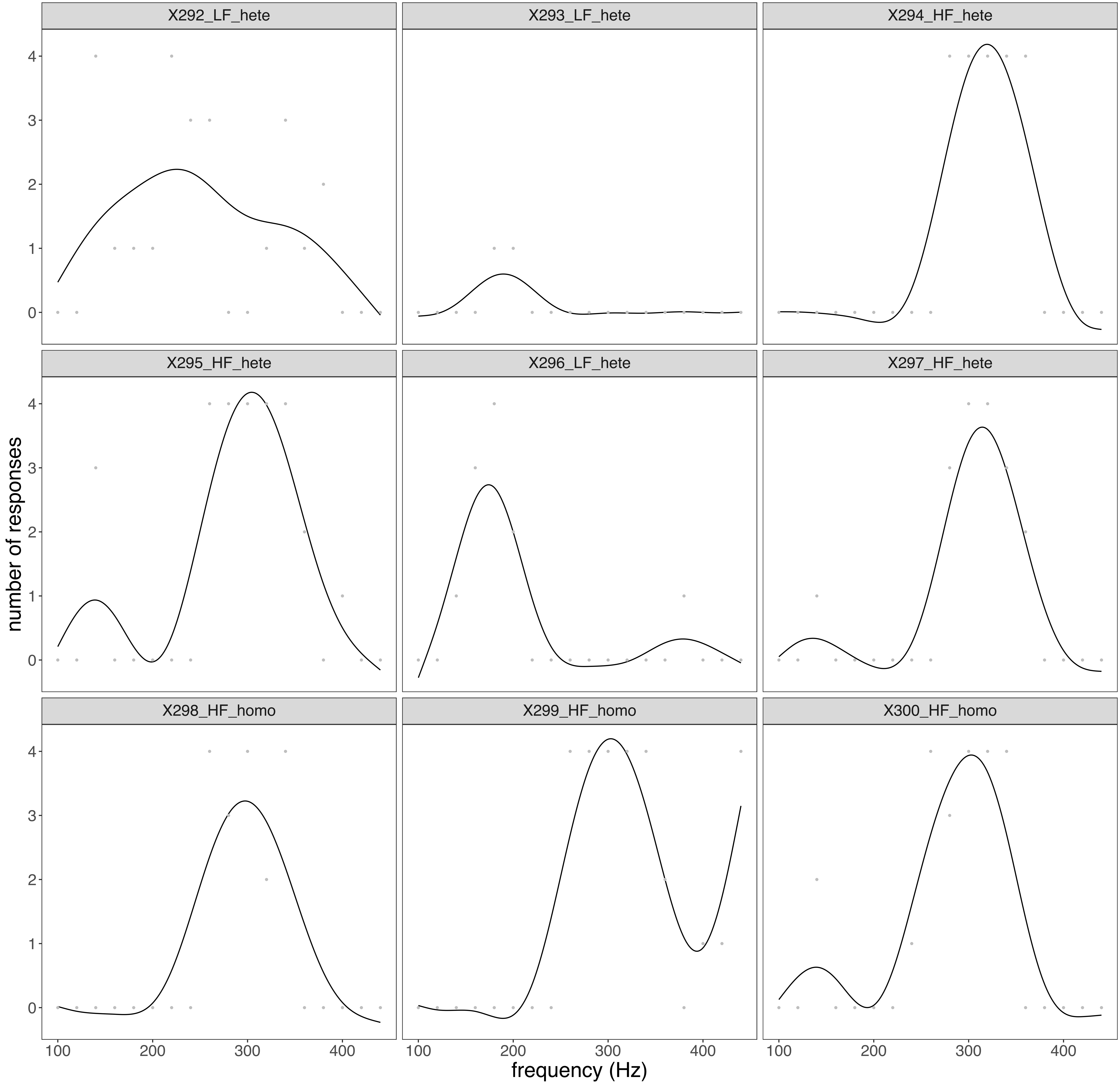
X285_LF_homo
X286_LF_homo
X287_LF_homo
X288_LF_homo
X289_HF_hete
X290_HF_hete
X291_HF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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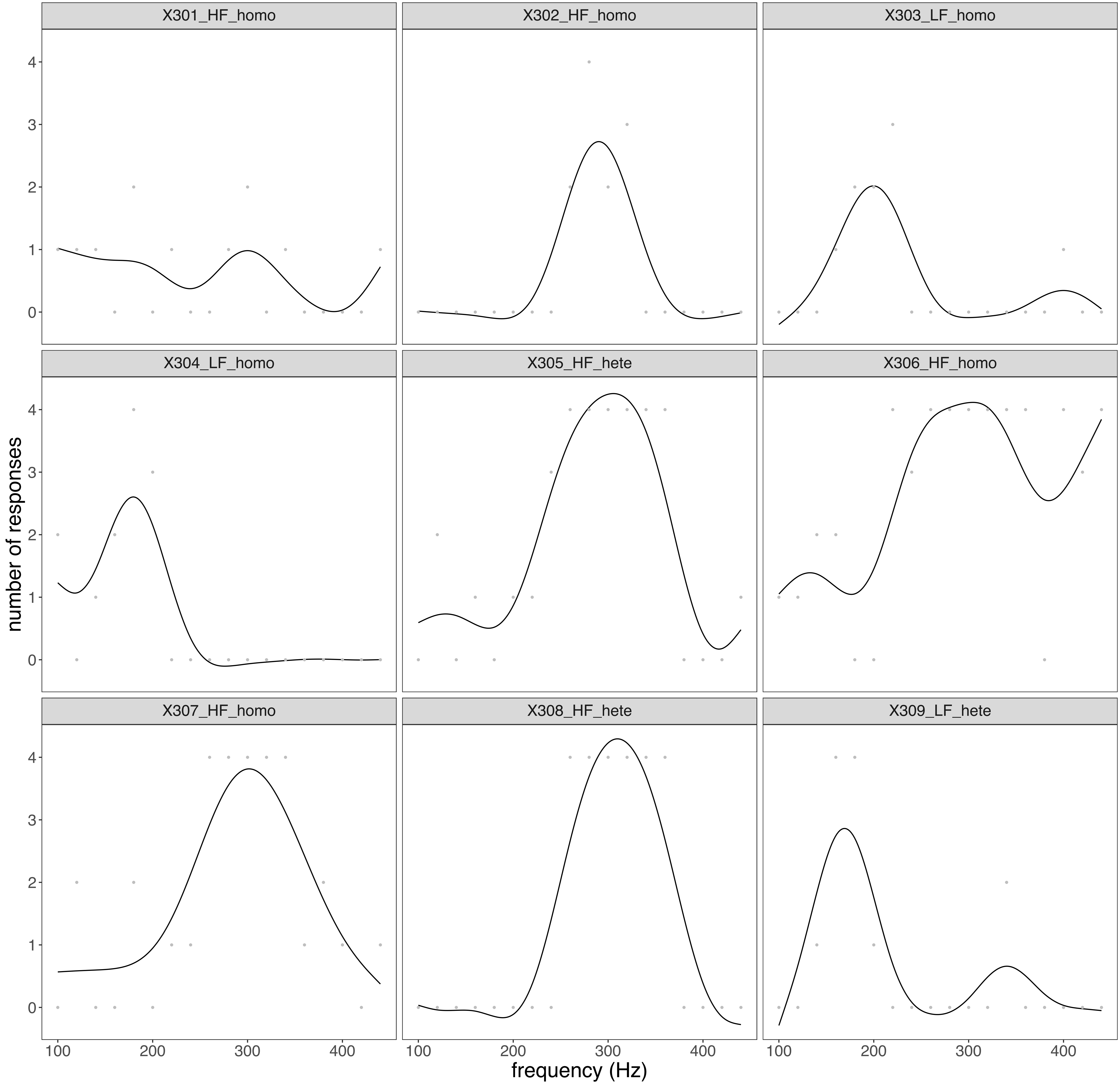
X295_HF_hete
X296_LF_hete
X297_HF_hete
X298_HF_homo
X299_HF_homo
X300_HF_homo
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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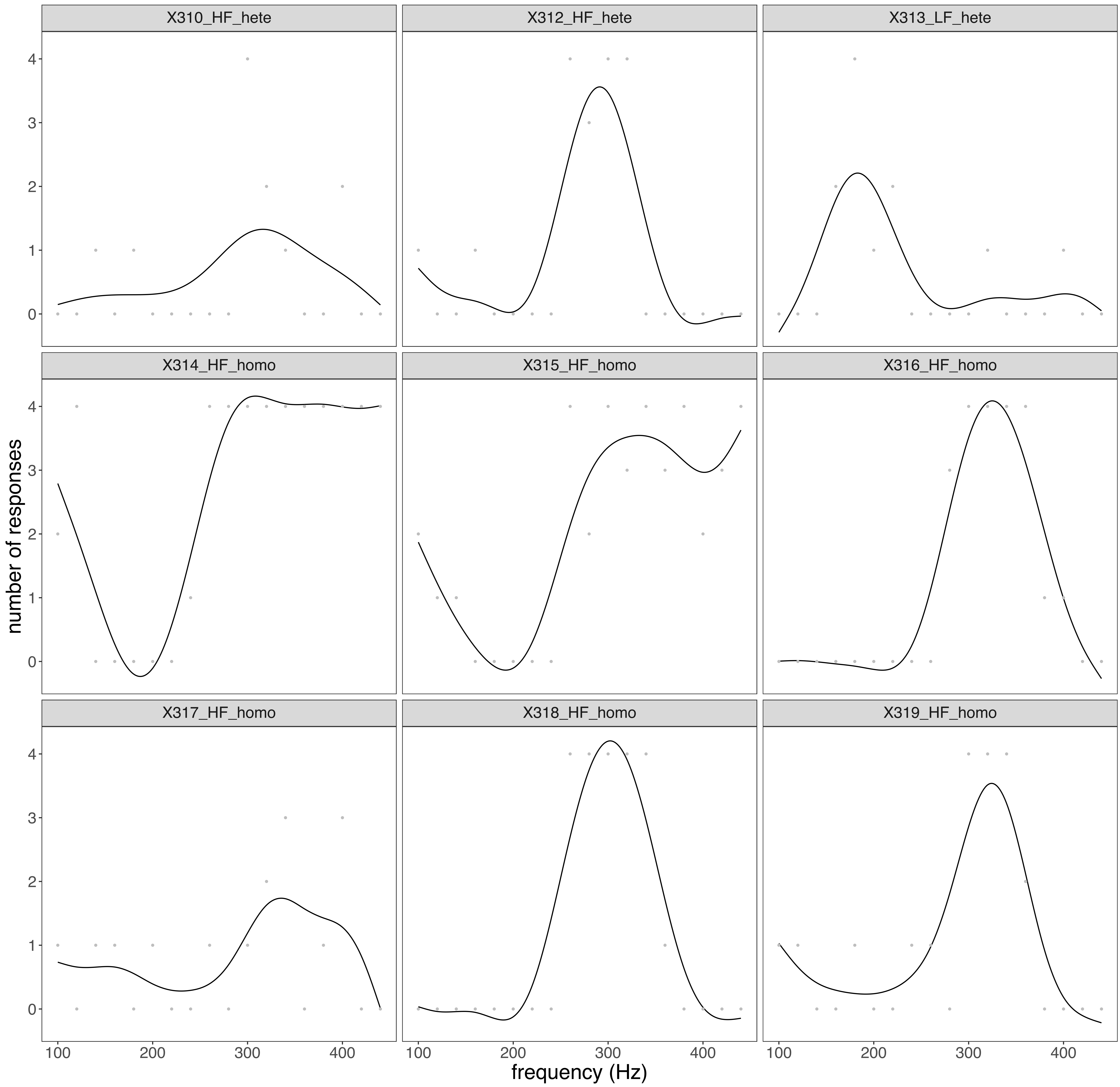
X304_LF_homo
X305_HF_hete
X306_HF_homo
X307_HF_homo
X308_HF_hete
X309_LF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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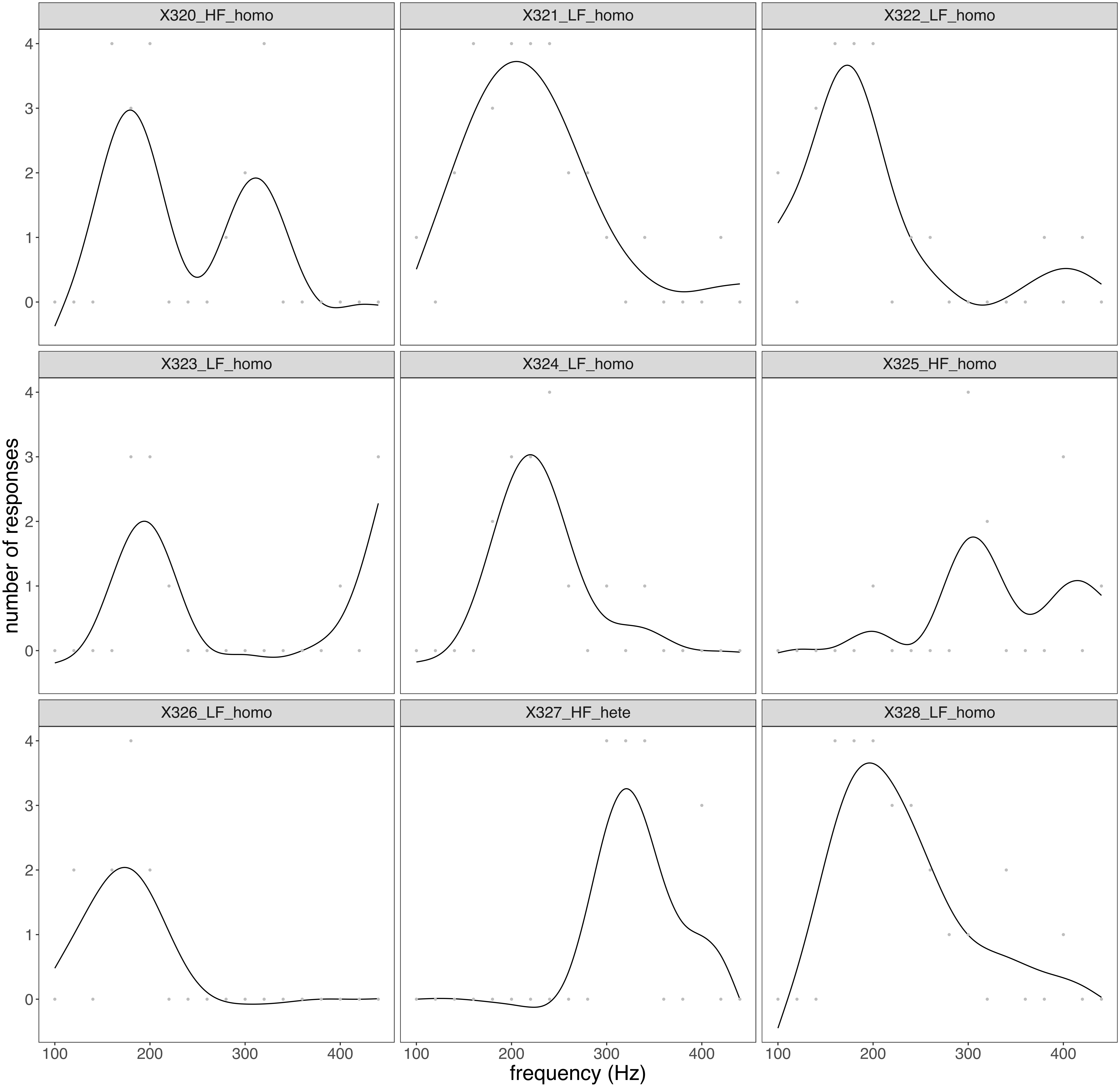
X313_LF_hete
X314_HF_homo
X315_HF_homo
X316_HF_homo
X317_HF_homo
X318_HF_homo
X319_HF_homo
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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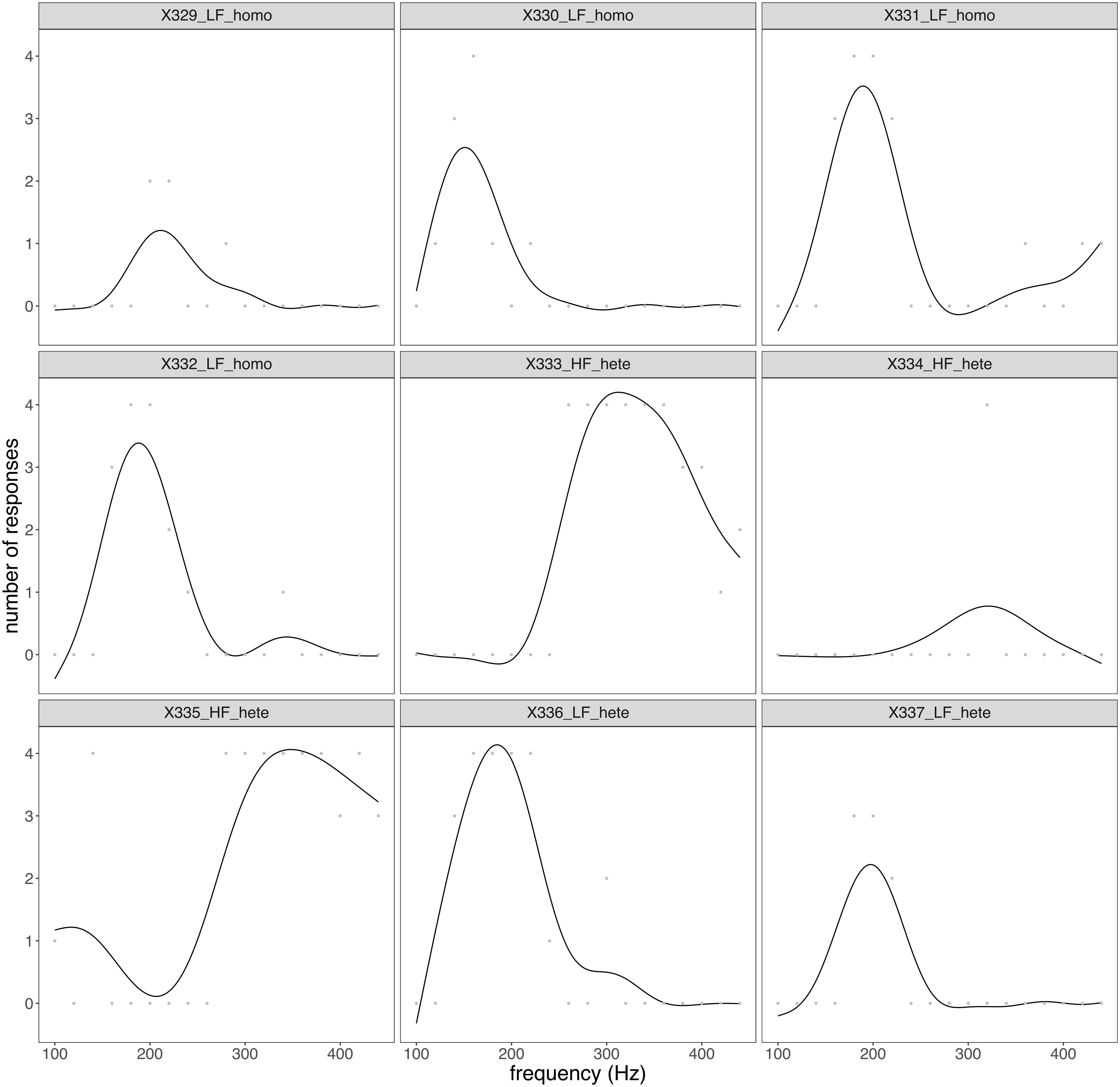
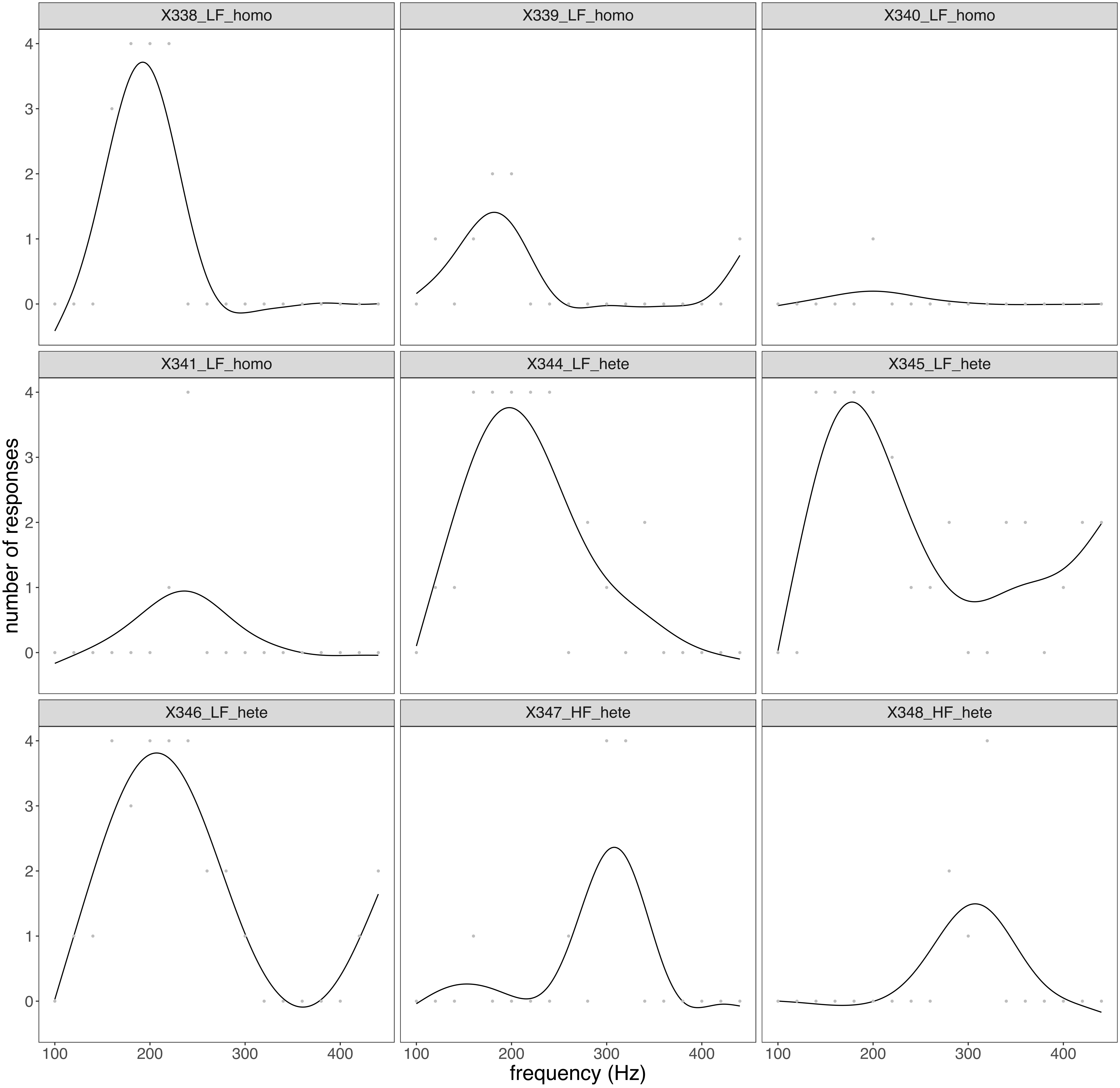
X321_LF_homo
X322_LF_homo
X323_LF_homo
X324_LF_homo
X325_HF_homo
X326_LF_homo
X327_HF_hete
X328_LF_homo
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400


X329_LF_homo
X330_LF_homo
X331_LF_homo
X332_LF_homo
X333_HF_hete
X334_HF_hete
X335_HF_hete
X336_LF_hete
X337_LF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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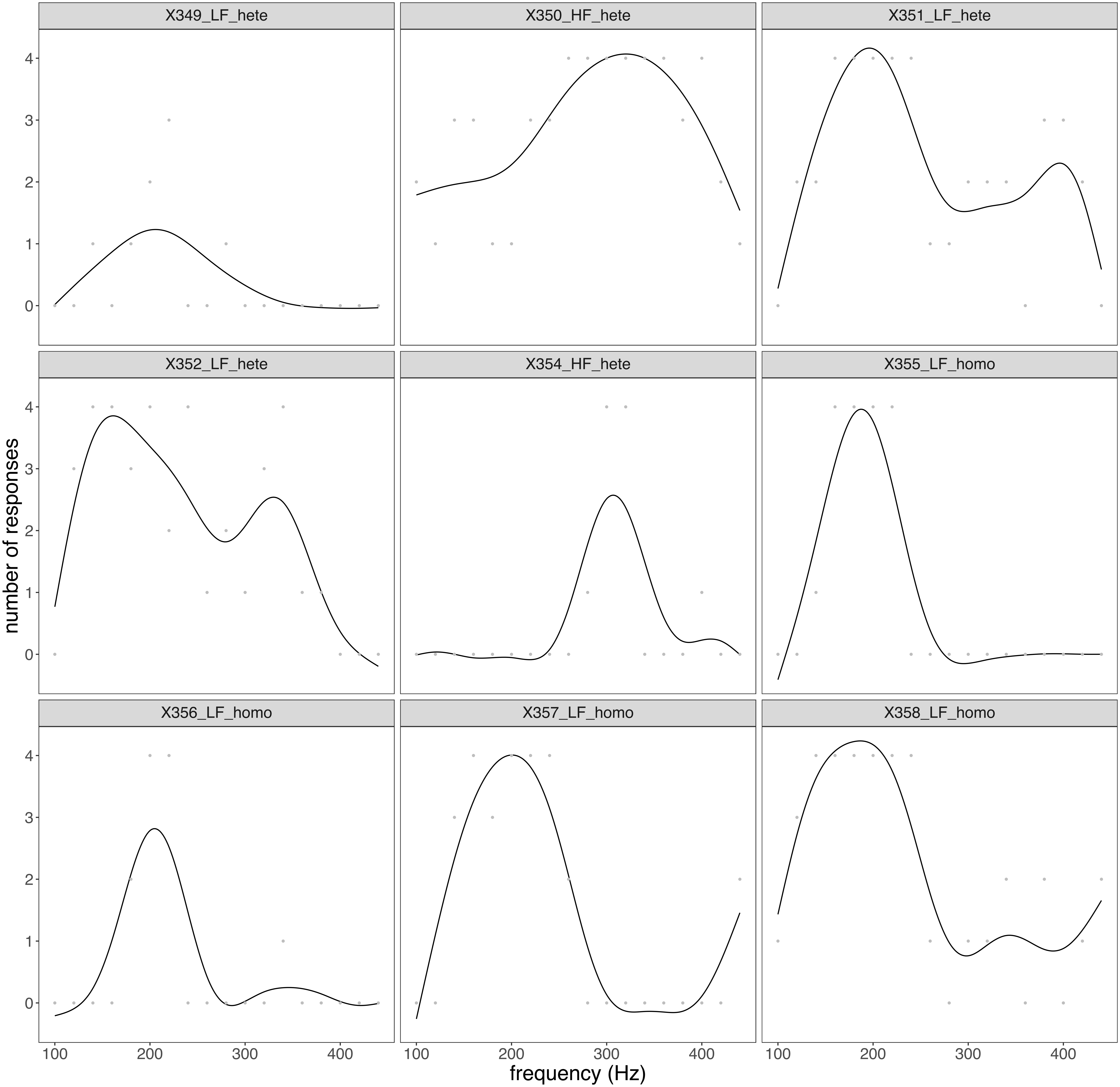
X340_LF_homo
X341_LF_homo
X344_LF_hete
X345_LF_hete
X346_LF_hete
X347_HF_hete
X348_HF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400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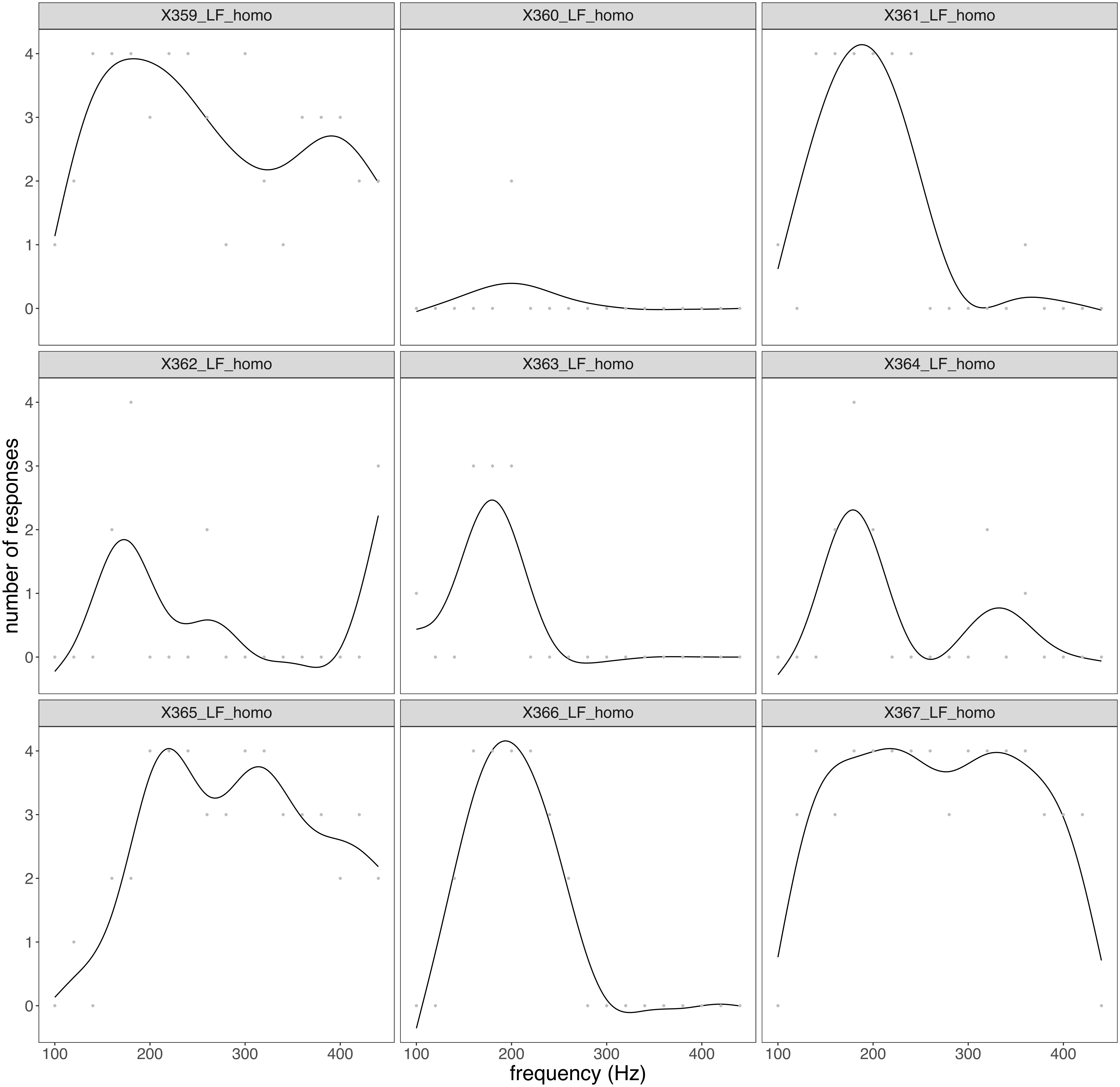
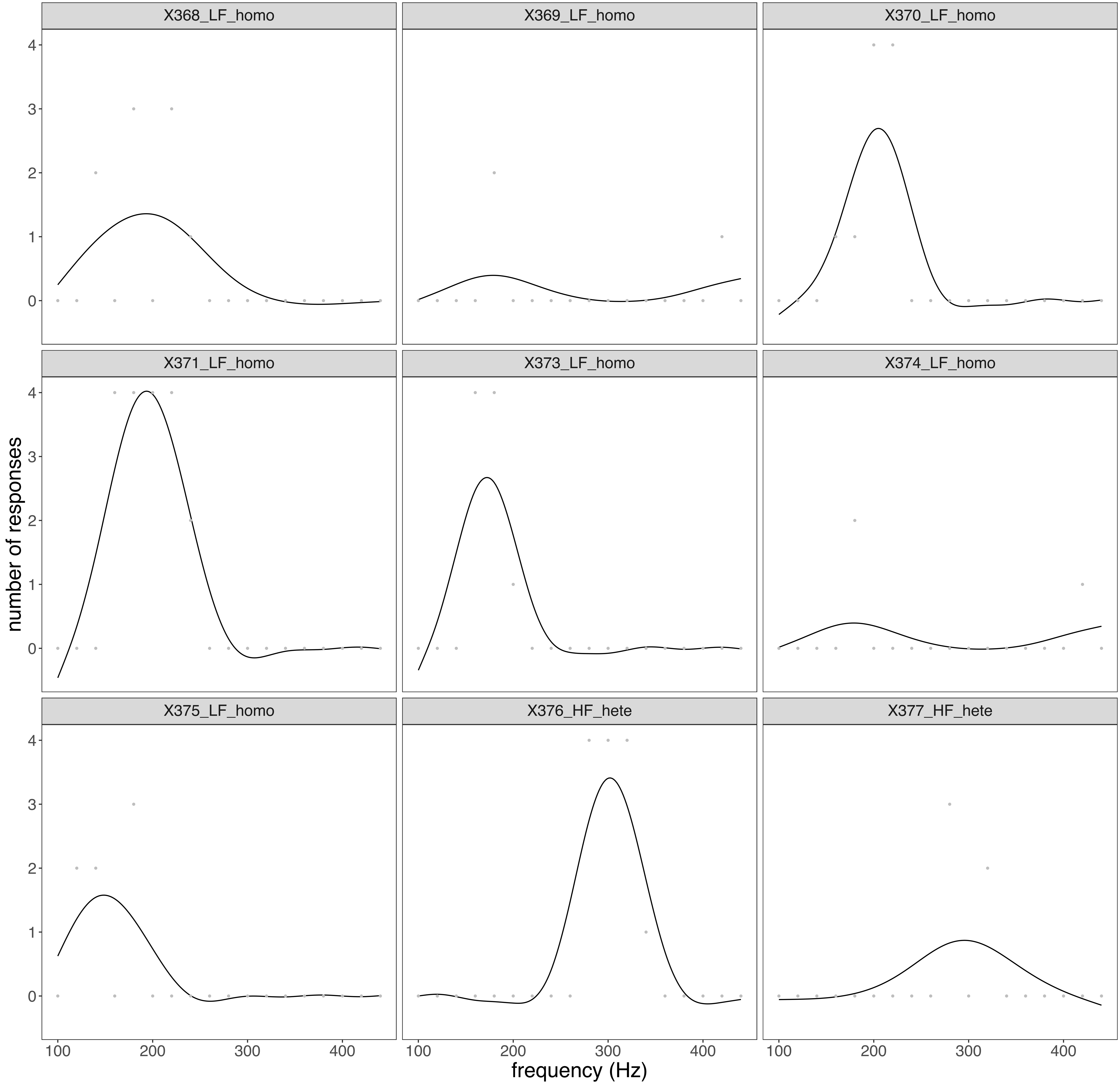
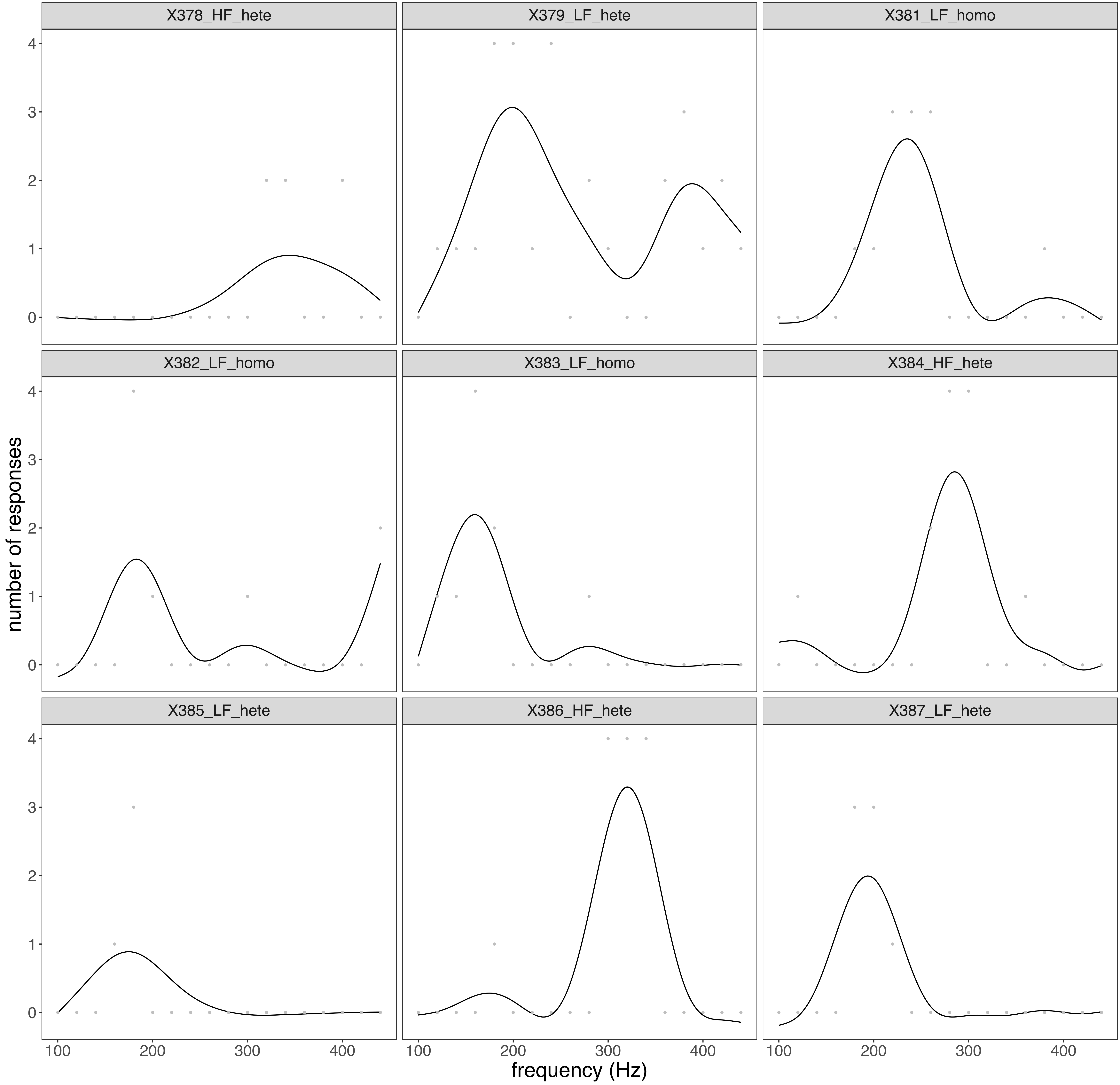
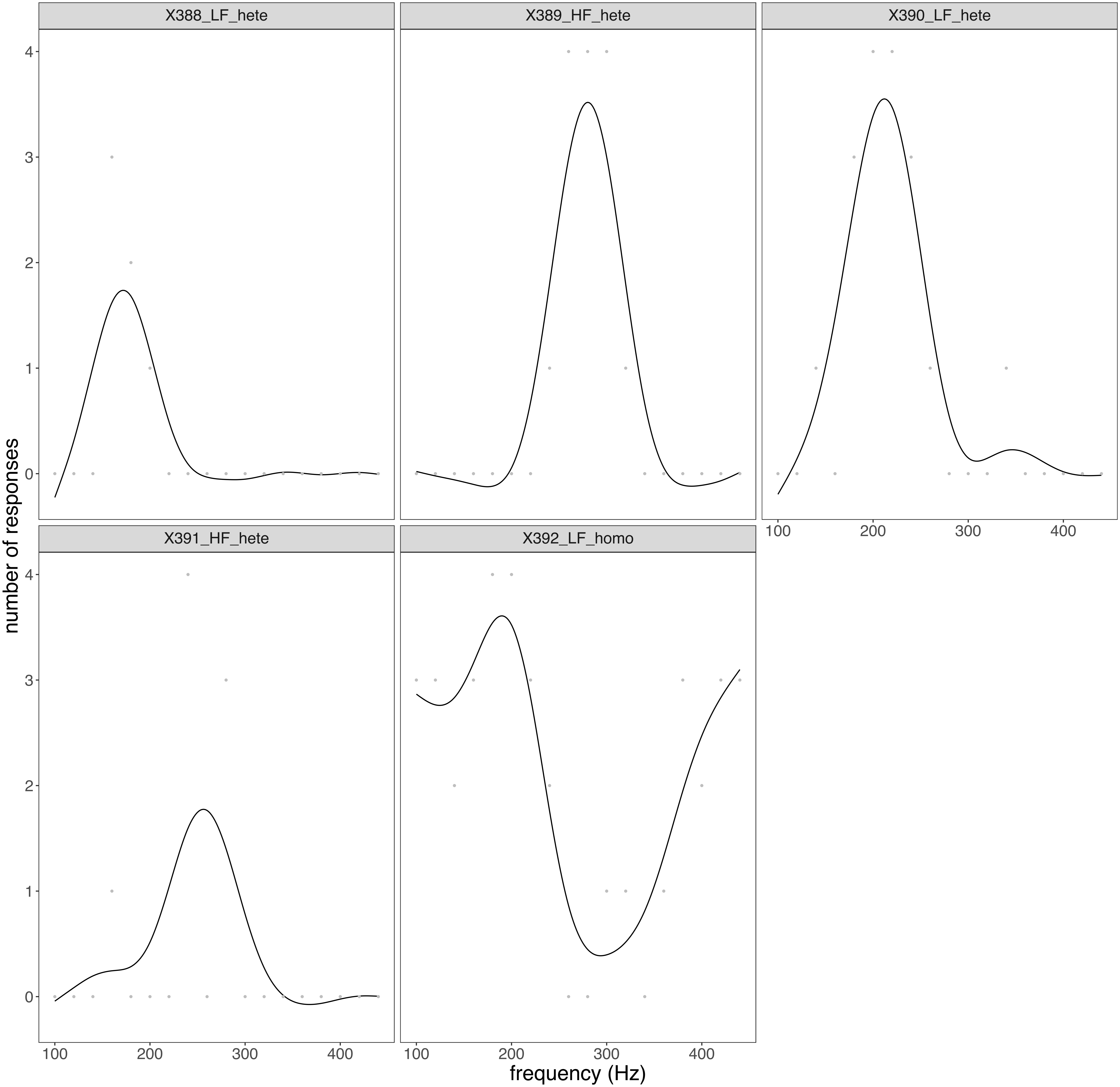
X350_HF_hete
X351_LF_hete
X352_LF_hete
X354_HF_hete
X355_LF_homo
X356_LF_homo
X357_LF_homo
X358_LF_homo
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400


X359_LF_homo
X360_LF_homo
X361_LF_homo
X362_LF_homo
X363_LF_homo
X364_LF_homo
X365_LF_homo
X366_LF_homo
X367_LF_homo
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400


X368_LF_homo
X369_LF_homo
X370_LF_homo
X371_LF_homo
X373_LF_homo
X374_LF_homo
X375_LF_homo
X376_HF_hete
X377_HF_hete
number of responses
frequency (Hz)
0
1
2
3
4
100
200
300
400


X378_HF_hete
X379_LF_hete
X381_LF_homo
X382_LF_homo
X383_LF_homo
X384_HF_hete
X385_LF_hete
X386_HF_hete
X387_LF_hete
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400


X388_LF_hete
X389_HF_hete
X390_LF_hete
X391_HF_hete
X392_LF_homo
number of responses
0
1
2
3
4
frequency (Hz)
100
200
300
400

# Social plasticity enhances signal-preference co-divergence

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[2] School of Biology, University of St Andrews, St Andrews, Fife KY16 9TH, UK

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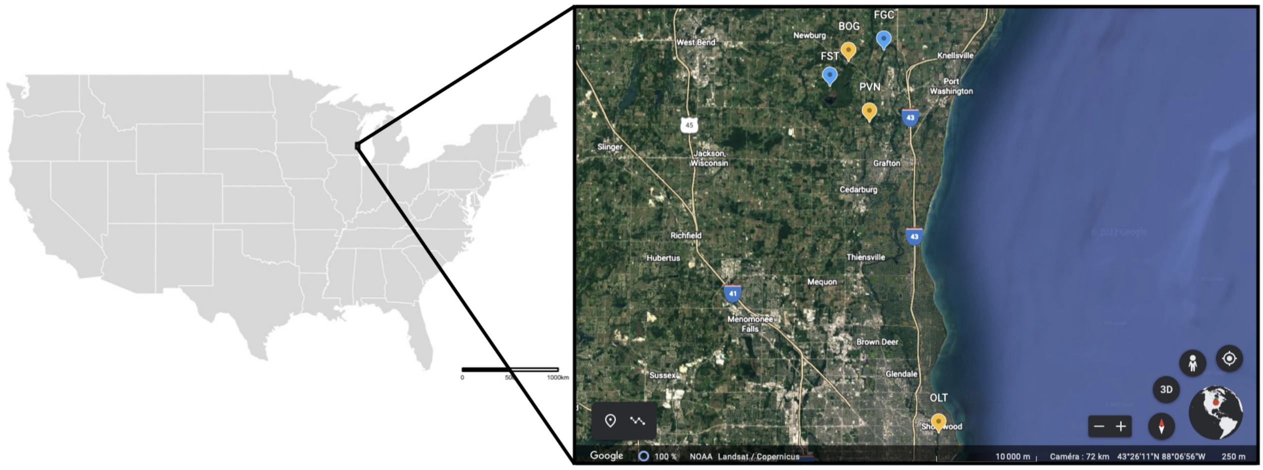

**Figure S1: Map of the sampling locations**. BOG, PNV and OLT are the allopatric locations in yellow. FST and FGC are the sympatric locations in light blue.

**Table S1: Replication levels for male and female *Enchenopa binotata* in different treatment, site and year.**

| Site $sp_{low}$ | Site $sp_{high}$ | Treatment | Years | Plant replicate | Nymphs | Males | Females |
|---|---|---|---|---|---|---|---|
| BOG | x | own | 2018-2020 | 9 | 360 | $sp_{low}$ 14 | $sp_{low}$ 51 |
| FGC | x | own | 2019-2020 | 8 | 320 | $sp_{low}$ 21 | $sp_{low}$ 41 |
| x | FGC | own | 2019-2020 | 5 | 200 | $sp_{high}$ 2 | $sp_{high}$ 15 |
| FST | x | own | 2018-2020 | 10 | 400 | $sp_{low}$ 10 | $sp_{low}$ 32 |
| x | FST | own | 2018-2020 | 7 | 280 | $sp_{high}$ 18 | $sp_{high}$ 17 |
| OLT | x | own | 2018 | 4 | 160 | $sp_{low}$ 7 | $sp_{low}$ 18 |
| PVN | x | own | 2020 | 4 | 160 | $sp_{low}$ 26 | $sp_{low}$ 27 |
| BOG | FST | mixed | 2018-2020 | 7 | 280 | $sp_{low}$ 6<br>$sp_{high}$ 6 | $sp_{low}$ 17<br>$sp_{high}$ 8 |
| FGC | FST | mixed | 2019-2020 | 7 | 280 | $sp_{low}$ 2<br>$sp_{high}$ 2 | $sp_{low}$ 18<br>$sp_{high}$ 18 |
| FST | FGC | mixed | 2019-2020 | 2 | 80 | $sp_{low}$ 1<br>$sp_{high}$ 1 | $sp_{low}$ 7<br>$sp_{high}$ 13 |
| FST | FST | mixed | 2018-2019 | 6 | 240 | $sp_{low}$ 10<br>$sp_{high}$ 3 | $sp_{low}$ 15<br>$sp_{high}$ 13 |
| OLT | FST | mixed | 2018 | 4 | 160 | $sp_{low}$ 7<br>$sp_{high}$ 2 | $sp_{low}$ 11<br>$sp_{high}$ 6 |
| PVN | FGC | mixed | 2020 | 2 | 80 | $sp_{low}$ 2<br>$sp_{high}$ 0 | $sp_{low}$ 6<br>$sp_{high}$ 3 |
| PVN | FST | mixed | 2020 | 5 | 200 | $sp_{low}$ 5<br>$sp_{high}$ 7 | $sp_{low}$ 20<br>$sp_{high}$ 18 |
|  |  |  | **Total** | 80 | 3200 | 153 | 375 |

**Table S2: Playback stimuli features for the two species.**

| **Signal trait** | ***sp**$_{low}$* | ***sp**$_{high}$* |
|---|---|---|
| Signal length (ms) | 950 | 710 |
| Pulse number | 3 | 4 |
| Pulse rate ($s^{-1}$) | 21 | 13.5 |
| Pulse length (ms) | 38 | 25 |
| Intersignal interval (ms) | 2841 | 3460 |
| Signals per bout | 4 | 4 |

**Table S3: Smoothing values for female preference curves.**

| **female number** | smoothing | treatment | species |
|---|---|---|---|
| 1 | 0,005 | homo | LF |
| 2 | 0,005 | homo | LF |
| 3 | 0,05 | homo | LF |
| 4 | 0,05 | homo | LF |
| 5 | 0,005 | homo | LF |
| 6 | 0,008787994 | homo | LF |
| 7 | 0,05 | homo | LF |
| 8 | 0,005 | homo | LF |
| 9 | 0,005 | homo | LF |
| 10 | 0,05 | homo | LF |
| 11 | 0,005 | hete | LF |
| 12 | 0,005 | homo | LF |
| 13 | 0,05 | homo | LF |
| 14 | 0,005 | homo | LF |
| 15 | 0,005 | homo | LF |
| 16 | 0,0348664 | homo | LF |
| 17 | 0,005 | hete | HF |
| 18 | 0,05 | hete | LF |
| 19 | 0,005 | hete | LF |
| 20 | 0,0289302 | hete | HF |
| 21 | 0,005 | hete | HF |
| 22 | 0,005 | hete | HF |
| 23 | 0,05 | hete | LF |
| 24 | 0,005 | hete | LF |
| 25 | 0,05 | homo | LF |
| 26 | 0,005 | homo | LF |
| 27 | 0,005 | homo | LF |
| 28 | 0,005 | homo | LF |
| 29 | 0,005 | homo | LF |
| 30 | 0,05 | hete | LF |
| 31 | 0,05 | hete | LF |
| 32 | 0,005 | homo | LF |
| 33 | 0,05 | homo | LF |
| 34 | 0,05 | homo | LF |
| 35 | 0,05 | homo | LF |
| 36 | 0,05 | homo | LF |
| 37 | 0,005 | homo | LF |
| 38 | 0,05 | homo | LF |
| 39 | 0,005 | homo | LF |
| 40 | 0,005 | homo | LF |
| 41 | 0,005 | hete | LF |
| 42 | 0,008933725 | hete | LF |
| 43 | 0,01640138 | hete | HF |
| 44 | 0,005 | hete | HF |
| 45 | 0,005 | hete | LF |

| | | | |
|---|---|---|---|
| 46 | 0,005 | hete | LF |
| 47 | 0,005 | hete | LF |
| 48 | 0,005 | hete | LF |
| 49 | 0,05 | hete | HF |
| 50 | 0,05 | homo | HF |
| 51 | 5,00E-04 | homo | LF |
| 52 | 0,005 | homo | LF |
| 53 | 0,05 | homo | LF |
| 54 | 0,05 | homo | LF |
| 55 | 0,005 | homo | LF |
| 56 | 0,04163705 | homo | LF |
| 57 | 0,005 | homo | LF |
| 58 | 0,001 | homo | LF |
| 59 | 0,005 | hete | LF |
| 60 | 0,005 | hete | LF |
| 61 | 0,005 | hete | LF |
| 62 | 0,05 | hete | HF |
| 63 | 0,005 | hete | HF |
| 64 | 0,05 | hete | LF |
| 65 | 0,005 | hete | HF |
| 66 | 0,005 | homo | LF |
| 67 | 0,01114964 | homo | LF |
| 68 | 0,005 | homo | LF |
| 69 | 0,005 | homo | LF |
| 70 | 0,005 | homo | LF |
| 71 | 0,006344236 | homo | LF |
| 72 | 0,01153259 | hete | LF |
| 73 | 0,005 | hete | HF |
| 74 | 0,005 | hete | LF |
| 75 | 0,005 | hete | HF |
| 76 | 0,005 | hete | HF |
| 77 | 0,005 | homo | LF |
| 78 | 0,005 | homo | LF |
| 79 | 0,02842651 | homo | LF |
| 80 | 0,03282305 | homo | LF |
| 81 | 0,05 | homo | LF |
| 82 | 0,005 | homo | LF |
| 83 | 0,006143665 | homo | LF |
| 84 | 0,005 | homo | LF |
| 85 | 0,005 | homo | LF |
| 86 | 0,005 | homo | LF |
| 87 | 0,03995623 | hete | HF |
| 88 | 0,005 | hete | LF |
| 89 | 0,005 | homo | LF |
| 90 | 0,02015132 | homo | LF |
| 91 | 0,005 | homo | LF |
| 92 | 0,005 | homo | LF |
| 93 | 0,01735497 | hete | LF |
| 94 | 0,01472007 | hete | HF |

| | | | |
|---|---|---|---|
| 95 | 0,005 | hete | LF |
| 96 | 0,005 | hete | LF |
| 97 | 0,005 | hete | LF |
| 98 | 0,05 | hete | LF |
| 99 | 0,005 | hete | LF |
| 100 | 0,005 | homo | HF |
| 101 | 0,005 | homo | HF |
| 102 | 0,005 | homo | LF |
| 103 | 0,005 | homo | LF |
| 104 | 0,005 | hete | LF |
| 105 | 0,05 | hete | HF |
| 106 | 0,005 | hete | LF |
| 107 | 0,05 | hete | HF |
| 108 | 0,005 | homo | LF |
| 109 | 0,001 | homo | LF |
| 110 | 0,005 | homo | LF |
| 111 | 0,005 | homo | LF |
| 112 | 0,005 | hete | HF |
| 113 | 0,05 | homo | LF |
| 114 | 0,005 | hete | LF |
| 115 | 0,005 | hete | HF |
| 116 | 0,01522379 | hete | LF |
| 117 | 0,005661009 | hete | HF |
| 118 | 0,005 | homo | HF |
| 119 | 0,005 | homo | HF |
| 120 | 0,05 | homo | LF |
| 121 | 0,005 | homo | LF |
| 122 | 0,005 | homo | LF |
| 123 | 0,005 | homo | LF |
| 124 | 0,005 | homo | LF |
| 125 | 0,005 | homo | LF |
| 126 | 0,05 | homo | LF |
| 127 | 0,008579381 | hete | LF |
| 128 | 0,005 | hete | LF |
| 129 | 0,008984747 | hete | LF |
| 130 | 0,005 | hete | LF |
| 131 | 0,005 | hete | LF |
| 132 | 0,05 | hete | LF |
| 133 | 0,00861887 | homo | HF |
| 134 | 0,05 | homo | HF |
| 135 | 0,005 | hete | HF |
| 136 | 0,005 | hete | LF |
| 137 | 0,005 | hete | LF |
| 138 | 0,05 | hete | LF |
| 139 | 0,05 | hete | LF |
| 140 | 0,05 | hete | HF |
| 141 | 0,008337663 | hete | LF |
| 142 | 0,005 | hete | LF |
| 143 | 0,005 | hete | LF |

| | | | |
|---|---|---|---|
| 144 | 0,006565031 | hete | HF |
| 145 | 0,0193478 | hete | HF |
| 146 | 0,005 | hete | LF |
| 147 | 0,01817277 | homo | LF |
| 148 | 0,05 | homo | HF |
| 149 | 0,005 | homo | LF |
| 150 | 0,005 | homo | LF |
| 151 | 0,005 | homo | LF |
| 152 | 0,005 | homo | LF |
| 153 | 0,02842651 | hete | LF |
| 154 | 0,005 | homo | HF |
| 155 | 0,01488127 | homo | HF |
| 156 | 0,05 | homo | HF |
| 157 | 0,005 | homo | LF |
| 158 | 0,02870255 | homo | LF |
| 159 | 0,005 | homo | LF |
| 160 | 0,03296894 | homo | LF |
| 161 | 0,005 | homo | LF |
| 162 | 0,005 | homo | LF |
| 163 | 0,005 | homo | LF |
| 164 | 0,01428094 | homo | LF |
| 165 | 0,005 | homo | LF |
| 166 | 0,005 | homo | LF |
| 167 | 0,005 | homo | LF |
| 168 | 0,005 | homo | LF |
| 169 | 0,005 | homo | LF |
| 170 | 0,008458835 | homo | LF |
| 171 | 0,005 | homo | LF |
| 172 | 0,005 | homo | LF |
| 173 | 0,005 | homo | LF |
| 174 | 0,05 | homo | HF |
| 175 | 0,005 | homo | HF |
| 176 | 0,005 | homo | LF |
| 177 | 0,005 | homo | LF |
| 178 | 0,05 | homo | LF |
| 179 | 0,005 | homo | LF |
| 180 | 0,005 | homo | LF |
| 181 | 0,05 | homo | LF |
| 182 | 0,01395171 | homo | HF |
| 183 | 0,02829996 | homo | HF |
| 184 | 0,005 | homo | HF |
| 185 | 0,05 | homo | HF |
| 186 | 0,005 | homo | LF |
| 187 | 0,005 | homo | LF |
| 188 | 0,005 | homo | LF |
| 189 | 0,005 | homo | LF |
| 190 | 0,02510162 | homo | LF |
| 191 | 0,005 | homo | LF |
| 192 | 0,005 | homo | LF |

| | | | |
|---|---|---|---|
| 193 | 0,04008847 | homo | LF |
| 194 | 0,02912358 | homo | LF |
| 195 | 0,005 | hete | LF |
| 196 | 0,007402557 | hete | LF |
| 197 | 0,005 | hete | LF |
| 198 | 0,005 | hete | LF |
| 199 | 0,05 | hete | LF |
| 200 | 0,005 | hete | LF |
| 201 | 0,005 | hete | LF |
| 202 | 0,005 | hete | HF |
| 203 | 0,005 | homo | LF |
| 204 | 0,0132327 | homo | LF |
| 205 | 0,005 | homo | LF |
| 206 | 0,005 | homo | LF |
| 207 | 0,005 | homo | LF |
| 208 | 0,005 | homo | LF |
| 209 | 0,005 | hete | HF |
| 210 | 0,005 | hete | LF |
| 211 | 0,001 | hete | LF |
| 212 | 0,05 | hete | HF |
| 213 | 0,005 | hete | LF |
| 214 | 0,005 | hete | HF |
| 215 | 0,007370676 | hete | HF |
| 216 | 0,005 | hete | LF |
| 217 | 0,005 | hete | LF |
| 218 | 0,005 | homo | LF |
| 219 | 0,007161673 | homo | LF |
| 220 | 0,005 | homo | LF |
| 221 | 0,005 | hete | HF |
| 222 | 0,005 | hete | LF |
| 223 | 0,005 | hete | HF |
| 224 | 0,006297506 | hete | HF |
| 225 | 0,005 | hete | HF |
| 226 | 0,02846831 | homo | LF |
| 227 | 0,005 | homo | LF |
| 228 | 0,005 | homo | LF |
| 229 | 0,005 | homo | LF |
| 230 | 0,005 | hete | HF |
| 231 | 0,01474261 | hete | HF |
| 232 | 0,005 | hete | HF |
| 233 | 0,005 | hete | LF |
| 234 | 0,05 | hete | LF |
| 235 | 0,005 | hete | LF |
| 236 | 0,005 | homo | LF |
| 237 | 0,005 | homo | LF |
| 238 | 0,005 | homo | LF |
| 239 | 0,02725187 | hete | LF |
| 240 | 0,005 | hete | HF |
| 241 | 0,05 | hete | HF |

| | | | |
|---|---|---|---|
| 242 | 0,005 | hete | HF |
| 243 | 0,005 | hete | LF |
| 244 | 0,005 | hete | LF |
| 245 | 0,01065837 | hete | HF |
| 246 | 0,01066943 | hete | HF |
| 247 | 0,005 | hete | LF |
| 248 | 0,005 | hete | HF |
| 249 | 0,008305693 | hete | HF |
| 250 | 0,005 | hete | LF |
| 251 | 0,005 | hete | LF |
| 252 | 0,05 | hete | LF |
| 253 | 0,005 | hete | LF |
| 254 | 0,005 | hete | HF |
| 255 | 0,005 | hete | HF |
| 256 | 0,005 | hete | LF |
| 257 | 0,005 | hete | LF |
| 258 | 0,05 | hete | HF |
| 259 | 0,007890447 | hete | HF |
| 260 | 0,005 | hete | HF |
| 261 | 0,01265992 | hete | HF |
| 262 | 0,04672711 | hete | HF |
| 263 | 0,005 | hete | LF |
| 264 | 0,005748247 | hete | LF |
| 265 | 0,005 | hete | HF |
| 266 | 0,005 | hete | HF |
| 267 | 0,01893001 | hete | HF |
| 268 | 0,005 | hete | LF |
| 269 | 0,005 | hete | LF |
| 270 | 0,005 | hete | LF |
| 271 | 0,0132224 | hete | HF |
| 272 | 0,05 | homo | LF |
| 273 | 0,005 | homo | LF |
| 274 | 0,005 | homo | LF |
| 275 | 0,005091953 | homo | LF |
| 276 | 0,005 | homo | LF |
| 277 | 0,005 | hete | HF |
| 278 | 0,005 | hete | HF |
| 279 | 0,005 | hete | HF |
| 280 | 0,05 | hete | LF |
| 281 | 0,005 | hete | LF |
| 282 | 0,005 | hete | HF |
| 283 | 0,005797086 | hete | HF |
| 284 | 0,005 | hete | LF |
| 285 | 0,005 | hete | HF |
| 286 | 0,0108547 | homo | HF |
| 287 | 0,005 | homo | HF |
| 288 | 0,005 | homo | HF |
| 289 | 0,005 | homo | HF |
| 290 | 0,005 | homo | HF |

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|---|---|---|---|
| 291 | 0,005 | homo | LF |
| 292 | 0,005 | homo | LF |
| 293 | 0,005 | hete | HF |
| 294 | 0,005 | homo | HF |
| 295 | 0,01689381 | homo | HF |
| 296 | 0,005381584 | hete | HF |
| 297 | 0,005 | hete | LF |
| 298 | 0,05 | hete | HF |
| 299 | 0,005 | hete | HF |
| 300 | 0,006644961 | hete | LF |
| 301 | 0,005 | homo | HF |
| 302 | 0,005 | homo | HF |
| 303 | 0,005 | homo | HF |
| 304 | 0,005 | homo | HF |
| 305 | 0,005 | homo | HF |
| 306 | 0,005 | homo | HF |
| 307 | 0,005 | homo | HF |
| 308 | 0,02014902 | homo | LF |
| 309 | 0,005 | homo | LF |
| 310 | 0,005 | homo | LF |
| 311 | 0,005 | homo | LF |
| 312 | 0,005 | homo | HF |
| 313 | 0,01429245 | homo | LF |
| 314 | 0,005 | hete | HF |
| 315 | 0,01022045 | homo | LF |
| 316 | 0,005 | homo | LF |
| 317 | 0,005 | homo | LF |
| 318 | 0,005 | homo | LF |
| 319 | 0,005 | homo | LF |
| 320 | 0,005428979 | hete | HF |
| 321 | 0,05 | hete | HF |
| 322 | 0,0172832 | hete | HF |
| 323 | 0,005073751 | hete | LF |
| 324 | 0,005 | hete | LF |
| 325 | 0,005 | homo | LF |
| 326 | 0,005 | homo | LF |
| 327 | 0,05 | homo | LF |
| 328 | 0,05 | homo | LF |
| 329 | 0,0209091 | hete | LF |
| 330 | 0,01208893 | hete | LF |
| 331 | 0,01382503 | hete | LF |
| 332 | 0,006074263 | hete | HF |
| 333 | 0,01893001 | hete | HF |
| 334 | 0,05 | hete | LF |
| 335 | 0,0346496 | hete | HF |
| 336 | 0,005 | hete | LF |
| 337 | 0,005 | hete | LF |
| 338 | 0,005 | hete | HF |
| 339 | 0,005 | homo | LF |

| | | | |
|---|---|---|---|
| 340 | 0,005 | homo | LF |
| 341 | 0,005653506 | homo | LF |
| 342 | 0,005 | homo | LF |
| 343 | 0,005 | homo | LF |
| 344 | 0,05 | homo | LF |
| 345 | 0,01194879 | homo | LF |
| 346 | 0,005 | homo | LF |
| 347 | 0,005 | homo | LF |
| 348 | 0,005 | homo | LF |
| 349 | 0,001 | homo | LF |
| 350 | 0,005 | homo | LF |
| 351 | 0,005 | homo | LF |
| 352 | 0,05 | homo | LF |
| 353 | 0,05 | homo | LF |
| 354 | 0,005 | homo | LF |
| 355 | 0,005 | homo | LF |
| 356 | 0,005 | homo | LF |
| 357 | 0,05 | homo | LF |
| 358 | 0,005 | homo | LF |
| 359 | 0,005 | hete | HF |
| 360 | 0,05 | hete | HF |
| 361 | 0,05 | hete | HF |
| 362 | 0,005 | hete | LF |
| 363 | 0,005 | homo | LF |
| 364 | 0,005 | homo | LF |
| 365 | 0,005 | homo | LF |
| 366 | 0,005 | hete | HF |
| 367 | 0,02842651 | hete | LF |
| 368 | 0,005 | hete | HF |
| 369 | 0,005 | hete | LF |
| 370 | 0,005 | hete | LF |
| 371 | 0,005 | hete | HF |
| 372 | 0,005 | hete | LF |
| 373 | 0,005 | hete | HF |
| 374 | 0,005 | homo | LF |

**Table S4: Variation in the variance of male signal frequency and female peak preference in two *Enchenopa* species according to own- vs. mixed-species rearing treatments.** Significant effects and marginally significant in bold.

| **Variance comparison** | **Term** | **F** | **Df (num, denom)** | **p** |
|---|---|---|---|---|
| Females | Peak preference of $sp_{low}$ in own- vs. mixed-species treatment | 1.43 | 168, 93 | **0.058** |
| | Peak preference of $sp_{high}$ in own- vs. mixed-species treatment | 3.40 | 31, 78 | **<<0.001** |
| Males | Signal frequency of $sp_{low}$ in own- vs. mixed-species treatment | 1.12 | 77, 30 | 0.76 |
| | Signal frequency of $sp_{high}$ in own- vs. mixed-species treatment | 1.19 | 19, 20 | 0.70 |

**Table S5: Results of generalized linear mixed model exploring the variation in the effect of rearing treatment on male signal frequency and female preference peak in one *Enchenopa* species (*$sp_{low}$*) according to population type (sympatric vs. allopatric)**. Significant effects (p-value < 0.10) are marked in bold.

| **Fixed effects** | | | |
|---|---|---|---|
| **Term** | $\chi^2$ | **Df** | **P-value** |
| Sex | 28.12 | 1 | **<0.0001** |
| Treatment | 0.04 | 1 | 0.84 |
| Population type (sympatric/allopatric) | 2.03 | 1 | 0.15 |
| Year | 9.72 | 1 | **0.0077** |
| Temperature | 6.41 | 1 | **0.011** |
| Sex × treatment | 0.87 | 1 | 0.35 |
| Treatment × population type | 0.10 | 1 | 0.75 |
| **Random effect** | | | |
| **Term** | $\chi^2$ | **Df** | **P-value** |
| Rearing plant/aggregation | 0.61 | 1 | 0.43 |

**Table S6: Results of generalized linear mixed model exploring the variation in the effect of rearing treatment on female preference strength in one *Enchenopa* species ($sp_{low}$) according to population type (sympatric vs. allopatric)**. Significant effects (p-value < 0.10) are marked in bold.

| **Fixed effects** | | | |
|---|---|---|---|
| **Term** | $\chi^2$ | **Df** | **P-value** |
| Population type | 0.02 | 1 | 0.88 |
| Treatment | 1.63 | 1 | 0.20 |
| Year | 0.53 | 1 | 0.77 |
| Temperature | 1.91 | 1 | 0.17 |
| Treatment × population type | 0.00 | 1 | 0.99 |
| **Random effects** | | | |
| **Term** | $\chi^2$ | **Df** | **P-value** |
| Rearing aggregation | 1.52 | 1 | 0.22 |