(As yet) unsolved questions about amniotic fluid-borne flavours and their perception by the human fetus. Reply to comments by Dr J. G. Alves

We thank Dr Alves for his comments on our paper entitled *"Flavor Sensing in Utero and Emerging Behaviors in the Human Fetus"* (Ustun et al., 2022). As we acknowledge and Dr Alves highlights, the behavioural responsiveness of the human fetus to chemosensory stimuli has so far been rarely investigated *in utero*, contrary to rat or ovine fetuses, which behavioural and psychophysiological reactions could be more extensively assessed using chemostimuli applied directly in the womb (e.g., Robinson et al., 1995; Schaal et al., 1991; Smotherman & Robinson, 1987). Rather most studies rely on *a posteriori* evidence with infants being re-exposed to chemostimuli which they could "only" have experienced prenatally. Therefore, many of the biological and psychobiological issues raised by Ustun et al.'s study need further work. Specifically, on 1) chemoreceptor system(s) and 2) brain structures and cognitive processes involved, on 3) the nature, kinetics and pathways of exogenous chemostimuli that reach fetal chemoreceptors, and on 4) the nature and timing of fetal responses. Our reply to Dr Alves's comments follows.

First, we need to clarify the form and cause of the facial responses in fetuses which might be a source of misunderstanding. First, in our study, maternal ingestion of a carrot capsule was *mostly* (but not exclusively) followed by fetal facial movements composing a "laughter-face" configuration, while a kale capsule *mostly* induced facial movements composing a "cry-face" configuration. Regarding facial configurations qualified as laughter-like, we coded 17 different facial movements frame-by-frame, 12 of which were analysed regarding their contributions to a laughter-face gestalt. As mentioned in our paper, the laughter-face gestalt involves, *but is not limited to*, the contraction of the cheek raiser and lip-corner puller muscles (FM6 and FM12, respectively; see Table 3 in Ustun et al. (2022) for the classification of facial gestalts). For example, the occurrence of cheek raiser (FM6) together

with tongue show (FM19) was also identified as a laughter-face gestalt. Thus, without the detailed and precisely timed coding of the facial gestalts of the fetuses, it is impossible to compare findings between our study and Dr Alves' own ultrasonographic on line observations. Dr Alves scanning of last-trimester fetuses, concludes that he could only rarely observe a laughter-face under stimulus-free conditions. Other investigators, however, report that fetuses do not so rarely display such laughter-like faces in the last trimester (e.g., AboEllail & Hata, 2017; Kawakami & Yanaihara, 2012; Kurjak et al., 2004; Reissland et al., 2011; Yigiter & Kavak, 2006). But we agree with Dr Alves that the frequency rate of observing the cheek raiser (FM6) as he pointed out are not that often observed. Previously it has been found that without stimulation, cheek raiser (FM6) can be observed at 32 and 36 weeks at a mean of 0.7 and 0.07 times per observation period respectively (in this case a 10 min period; see Reissland et al., 2016, for a detailed account). When overtly stimulating the fetus, we obtained very different results from non-stimulated fetuses, as noted in our study: fetuses exposed to flavour stimuli (kale or carrot) showed a significantly higher frequency of fetal facial reactions per min compared to the control group fetuses who were not exposed to any of these experimental flavours.

Second, Dr Alves compares our fetal results to results obtained from facial response to sweet stimuli in preterm or term newborn infants. However, our data are not comparable to Rosenstein & Oster (1988) or Zhang & Li H-qi (2007) who observed face responses of newborns being stimulated with more or less concentrated sweetness delivered with confounded tactile stimuli to the lips and tongue. Also, we did not claim that carrot flavour was "sweet", rather we refer to "non-bitter". A complex carrot flavour infused physiologically into the womb is very different from giving newborns pure sucrose in co-stimulation with oral somesthesis. Acknowledging this, we did not identify carrot flavour as "sweet," but rather "non-bitter".

Regarding the claim of possible chemosensory interferences between the experimental carrot/kale flavours and residual flavours from earlier, uncontrolled intakes by mothers, we agree that the extremely complex and fluctuating chemical ecology of the amniotic fluid is not yet well researched. Flavours from successive maternal intakes may indeed overlap in the amniotic fluid, potentially leading to a swamping of the amniotic chemosphere that may abolish or at least attenuate fetal sensation and responsiveness through processes of chemosensory adaptation or habituation. This adaptation or habituation to certain flavours, however, will probably be reversed by fresh chemosensory inputs caused by another maternal intake, by fetal inhaling/swallowing activity replenishing the amniotic fraction that contacts chemoreceptors, or by both events occurring in synchrony. In this context, the one-hour fasting period in our study, although unable to eliminate latent amniotic flavours due to earlier inputs, aimed to standardize a relatively stable background, and thereby to eliminate any likely increase of dishabituating chemostimuli within the 45-minute ultrasound scan sessions.

Dr Alves suggested, based on one study, that the mother-to-fetus transfer of flavour compounds would be around 45 minutes when counting the time between maternal garlic ingestion and adult perceptible amniotic fluid odour change (Dr Alves cited Beauchamp & Mennella, 2011 for this information incorrectly; it was reported in Mennella et al., 1995). The time suggested corresponds to our timing of the ultrasound scan which starts approximately 20 minutes after maternal ingestion of a flavour capsule followed by approximately 25-minute ultrasounds scan of the fetal face. The recorded time-window of fetal response monitoring was thus about 45 minutes, beginning at mothers' intake. Under these conditions, we observed the first fetal reactions around 30 minutes after the mothers' ingestion of the carrot/kale flavours. Although not much is known about the biotransformation and transplacental kinetics of flavours ingested by pregnant women, the relatively short transfer

time and fetal reaction reported is compatible with pharmacokinetic data on metabolites (e.g., Hay, 1994; Battaglia, 2002), drugs (e.g., Szeto, 1993), or other xenobiotics (e.g., Codaccioni et al., 2019) in the materno-placento-fetal system. Furthermore, the evidence cited by Dr Alves (Mennella et al., 1995) is based on adults smelling the odour of amniotic fluid collected 45 min after maternal ingestion of a garlic capsule. But given that adults are less sensitive to smells of given odorants compared to neonates (see Loos et al., 2014), we can arguably infer that fetal olfaction *in utero* might be more sensitive than that of adults (e.g., Schaal et al., 1995). Therefore, we suggest given that perinates effectively sense chemostimuli at lower detection thresholds than adults, they will react at earlier timepoints than adults to the increasing concentration of the flavour in their amniotic fluid.

To reach fetal chemoreceptive structures and induce fetal responses, the flavour content of the swallowed capsules must be absorbed into the maternal bloodstream, metabolised and circulated through the placenta and fetus, and collected in the amniotic fluid. However, before the flavour compounds gather in the amniotic pool, the pulmonary flow of amniotic fluid can attain the diffuse chemosensory system distributed in the lungs and larynx (e.g., Behrens & Meyerhof, 2011), nasal chemoreception via the retronasal pathway, and oral chemoreception. Likewise, the steps of fetal metabolism and amniotic collection might be by-passed by the haematogenic pathway by which flavour molecules reach the fetal gustatory or olfactory neuroepithelia (as first suggested by Bradley & Mistretta, 1975). Blood-borne flavours can indeed activate olfaction/taste by diffusion from the capillaries that irrigate the chemosensory neuroepithelia, and odour/taste learning can even be engaged in this way as demonstrated in adult rats (e.g., Bradley & Mistretta, 1971; Kasama & Zusho, 1981; Maruniak et al., 1983a, b). Therefore, much like oxygen and nutrients, flavour molecules may similarly reach the fetal odour and/or taste receptors when they diffuse from the capillaries that lay adjacent to the olfactory sensory neurons or taste buds (Schaal et al., 1995). The

capillary network within the olfactory neuroepithelium is indeed particularly dense during fetal development (Sangari et al., 2000). In sum, once flavourants ingested by the mother pass beyond the placenta, several pathways of chemostimuli to chemosensation may function sequentially, the potential vascular route and the retronasal route channelling the amniotic fluid originating in the lungs being briefer than the more intuitive way based on the orthonasal inhalation of amniotic fluid. These proposed multiple routes to the fetal disponibility of flavours ingested by the mother might explain the relatively fast fetal reactions observed in the current study.

Regarding the claim that auditory or tactile stimulation might have affected fetal facial movements, to the best of our knowledge, there are only a few studies showing that maternal touch and sound might affect fetal facial reactions using 4D ultrasound scanning (Marx & Nagy, 2015; Nagy et al., 2021). For example, a recent study showed that the "duration of mouth opening" was higher when there is an interactive talk compared to non-interactive talk and the "duration of sucking" was higher when there is an interactive touch compared to non-interactive touch (Nagy et al., 2021). Our experiment was performed in a quiet dimly lit room where mothers were encouraged to observe their fetus on the screen but did not talk during the scan, nor did they touch their abdomen which was covered with ultrasound gel. Neither the sonographers who were blinded to the hypotheses and participant group allocation, nor the experimenter talked to each other during the scan. The sonographer talked during the scan infrequently to instruct mothers to lie on one side or back. Before and after the scan, the experimenter gave instructions and/or answered questions by talking to the mothers, but there was no communication between them during the scan. Since there was no interactive touch and/or sound condition in our study, we expect such variables will unlikely affect our results.

In conclusion, we are grateful for Dr Alves's interest and affirmation for "the need for further studies on this relevant topic" and hope that we have clarified his questions on the study. Regrettably, the *non-invasive* delivery of chemostimuli to human fetuses will probably remain irreducibly imprecise in temporal terms. But, despite such temporally imprecise - but ecologically valid - conditions of stimulation, our study showed discriminative facial reactions of 32-to-36-gestational-week fetuses following a relatively short delay after maternal ingestion of flavour compounds. To note, although we used convenience terms relating to emotional behaviour to describe the facial responses of the fetuses, we cannot yet infer fetal emotional states. But we have shown in this, and many previous studies, that human fetuses are capable of structured facial reactions which parents interpret as emotional expressions.

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