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Mild maternal stress disrupts associative learning and increases aggression in offspring

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ABSTRACT

Maternal stress has been shown to affect behaviour of offspring in a wide range of animals, but this evidence has come from studies that exposed gestating mothers to acute or severe stressors, such as restraint or exposure to synthetic stress hormones. Here we show that exposure of mothers to even a mild stressor reduces associative learning and increases aggression in offspring. Female guppies were exposed to routine husbandry procedures that produced only a minimal, non-significant, elevation of the stress hormone cortisol. In contrast to controls, offspring from mothers that experienced this mild stress failed to learn to associate a colour cue and food reward, and showed a greater amount of inter-individual variation in behaviour compared with control offspring. This mild stress also resulted in offspring that were more aggressive towards their own mirror image than controls. While it is possible that these results could represent the transmission of beneficial maternal characteristics to offspring born into unpredictable environments, the potential for mild maternal stress to affect offspring performance also has important implications for research into the trans-generational effects of stress.

Introduction

There are many ways that parents can influence offspring phenotype through non-genomic pathways both directly (e.g. nutrient and hormone transfer) and indirectly (e.g. parental care behaviours) (Bernardo, 1996; Donaldson et al., 2008a,b; Mousseau and Fox, 1998). While in some species these parental effects may be due to both parents, maternal effects on offspring have received the most attention and have been demonstrated in both oviparous and viviparous animals. A key element of non-genomic maternal influence is maternal stress and there is considerable evidence in mammals that exposure to pre-natal stress affects the hormonal and behavioural development of offspring (Huizink et al., 2004; de Kloet et al., 2005; Kofman, 2002). In particular, gestational stress can increase the incidence of anxiogenic and depressive-like behaviours in offspring and impair cognitive performance (Vallée et al., 1999; Weinstock, 2005).

The majority of studies investigating maternal stress and offspring performance have subjected mothers to significant levels of stress, either through direct imposition of an acute stressor or by supplementation of endogenous hormone levels with synthetic hormones (Eriksen et al., 2006; Janczak et al., 2006; Sloman, 2010; Takahashi et al., 1998; Vallée et al., 1999; Weinstock, 2005). However, the effect of milder stressors, which are likely to be a more common experience for a mother, has received little attention. Transmission of a variety of information about a mother's environment to her offspring has been previously demonstrated (Dadda and Bisazza, 2012; Giesing et al., 2011), possibly through the involvement of glucocorticoid hormones, although the exact mechanisms remain elusive. Thus, it seems feasible that mild environmental disturbance could alter offspring physiology and behaviour with implications for our interpretation of non-genomic effects.

The aim of this study was to examine the effects of mild maternal stress induced on offspring in the viviparous guppy. To our knowledge this is the first study to investigate the effects of a mild maternal husbandry stressor in any animal and the first to consider the effects of maternal stress in a viviparous fish. Maternal stress was applied both prior to fertilisation and during gestation. Offspring behaviour was tested using measures of associative learning and competitive ability.

Methods

Adult virgin female guppies (total length 4.51 ± 0.9 cm), *Poecilia reticulata*, of various colour morphs were obtained from three different home aquarium fish suppliers and were kept in high density 10 l (21.5 × 19.5 × 40 cm) stock tanks (~1.5 g l^{-1}) on 200 l recirculating systems (flow rate: 0.6 l min^{-1}; 26.9 ± 0.1 °C; pH 6.5 ± 0.1,

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dissolved oxygen 7.52 ± 0.02 mg l^{-1}, 12:12 h light:dark) for 4 weeks prior to the start of the study. Fish were fed to satiation daily with a diet supplied by WALTHAM Centre for Pet Nutrition (http://www.waltham.com/). We used fish from three different suppliers to ensure that any significant effects were repeatable in different varieties of guppy and not restricted to one particular batch or strain of fish. Different sources of fish were included as 'tank replicates' in data analysis.

Groups of six virgin females were then assigned to either a control or stress treatment (n = 3 groups of six females per treatment, i.e. 36 females in total) and held in 19 l tanks on the recirculating system with a plastic plant as environmental enrichment. Each replicate consisted of fish from a different fish supplier. Typical home aquaria husbandry procedures were used to induce mild maternal stress and were carried out twice weekly for 4 weeks. Females assigned to the control treatment experienced 2 min of tank siphoning, where a siphon was used to remove waste food and faeces from the tank, along with a small amount of water. During siphoning, water was continuously replaced via the recirculating system and care was taken to disturb the fish as little as possible. In the stress treatments, tanks were siphoned for 2 min, fish were then carefully netted and transferred to a plastic tank containing 2 l of water from their home tank system for a further 2 min. During this time, the plastic plant in their home tank was moved to a new location. Fish were then re-netted and returned to their original tank. This stress treatment was based on typical home husbandry procedures for pet fish tanks determined via a survey of fish owners (Sloman, K. unpublished data) where fish are removed from their tank while the home tank is thoroughly cleaned.

After 4 weeks, three randomly chosen males of various colour morphs (orange delta, yellow delta, blue delta, black delta and/or snakeskin delta morphs) were introduced into each tank. These males were allowed 3 days to interact and inseminate the females, at which time they were removed and another set of three males were introduced for a further 3 days. Females were allowed to multiple mate as this reduces gestation time and produces larger broods in comparison to singly-mated females (Evans and Magurran, 2000). Husbandry treatments continued post-mating as previously detailed, but their frequency was reduced to once weekly to prevent gestating females from selectively aborting broods (Evans and Magurran, 2000).

After three weeks of gestation, females were easily identifiable due to their size as being in the latter stages of pregnancy. Treatments were then stopped and females were held in nylon breeding nets (3 mm mesh size) in their groups of six. Fry were born 21–27 days after the males had been removed. Mothers and breeding nets were then removed from the tanks and offspring were raised in situ (n = 3 tanks of offspring per treatment). Fry were fed a mixture of live *Artemia*, a commercial flake food (Aquarian) and high protein pelleted food (ZM200) daily. Six juveniles were randomly selected from each replicate for plus-maze trials at 14 weeks of age (i.e. n = 18). At 18 weeks of age, when fish were reproductively mature, six individuals not previously used in the plus-maze trials were selected for mirror-image tests (i.e. n = 18).

Water-borne cortisol sampling

To determine the effect of the husbandry stress treatments on levels of cortisol, an additional experiment was carried out. A new group of female guppies were acclimated to tank conditions in the same way as before and water-borne cortisol was sampled after one exposure to the stress treatments. Water cortisol concentrations following the control and experimental husbandry treatments were compared with water cortisol released from guppies following an acute 1 h transport stress which represented a transport stress from pet shop to home. For the measurements of water cortisol following husbandry procedures, females were removed from their tanks and placed in groups of three (n = 16 groups of three per husbandry treatment) into a beaker containing 150 ml of clean water for 30 min based on the methods of Sebire et al. (2007). For the acute transport stress, groups of guppies (n = 9 groups of three) were held in a plastic bag containing 150 ml water, placed inside a dark box, and moved around the laboratory for 1 h prior to cortisol measurements. After this time, water cortisol concentration was extracted according to the methods of Ellis et al. (2004) and measured using a commercial ELISA (DRG Instruments). The commercial ELISA used a cortisol antibody sensitive to 2.5 ng ml^{-1} (6.9 nmol l^{-1}), the average intra and inter assay variation was 5.6% and 5.9% respectively. Blank samples of tank system water were used to account for background concentrations of cortisol. All stressors and cortisol measurements were conducted at the same time of day to control for circadian fluctuations in endocrine status.

Offspring behavioural tests

Plus-maze test

The four-armed radial plus-maze (10 l) supplied with system water (26.9 ± 0.1 °C), was based on the design of Sison and Gerlai (2010) and made of white, opaque polypropylene. Each arm was 30 cm long, 10 cm wide and 10 cm high and arms were connected by a central 10 × 10 cm central square. At the end of each individual arm there was a section of tubing connected to an external syringe loaded with bloodworm. Fish were held for 10 s within the central square in a 'start-box', an open-topped transparent container attached to a pulley system to acclimate to the maze environment before the start-box was remotely removed.

Fish were subjected to five habituation trials in the plus maze, one per day on consecutive days, in decreasing group sizes and times. In the first of these trials, six fish were placed into the start-box, in single treatment groups, and then allowed to explore the plus-maze for 1 h, with food released from all arms after 30 min. The second trial allowed groups of three fish to explore the maze for 30 min with food administered after 15 min in all arms. The third allowed pairs of fish to explore the maze for 20 min with food administered after 10 min and the fourth and fifth, single fish to explore the maze alone for 10 min with food administered in all arms after 5 min. Fish were fed at no other time other than within the plus-maze.

After habituation trials, each fish spent 10 min individually within the maze twice daily for 10 days, once in the morning and once in the afternoon. Each period within the plus-maze was recorded from above using a mounted camcorder (Panasonic SDR-S50). A large piece of red card with a hole in the centre was placed at the end of one arm with the food-dispensing tubing pushed through. Food was administered after 5 min of fish being within the plus-maze and only from this colour-coded arm (target-arm), never from non colour-coded arms (non-target arms). All arms, target and non-target, were loaded with bloodworm to ensure that any scent of food leaked equally from each. The plus-maze was curtained with black cloth to prevent any external features acting as guides to the target arm. The order in which fish were subjected to plus-maze trials and the orientation of the target-arm was randomised each day.

Maze performance was analysed using JWatcher (http://www.jwatcher.ucla.edu/). The percentage of time spent within the target arm and the percentage of entries made to the target arm were recorded for 10 min after the fish was released from the start-box. Arm entries were recorded as soon as a fish entered that specific arm, if a fish exited an arm and remained within the central square, it was considered to still be within the last arm visited until it entered a different arm. Each fish completed the plus-maze 20 times over a period of 10 days.

Mirror image test

Mirror image tests have previously been used as a measure of competitive ability since fish respond to their mirror image as if it were another individual (Brick and Jakobsson, 2002; Sloman and Baron, 2010). At approximately 18 weeks of age, mature single males (n = 18) from each maternal treatment were randomly selected for