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RESEARCH ARTICLE

Fear is the mother of invention: anuran embryos exposed to predator cues alter life-history traits, post-hatching behaviour and neuronal activity patterns

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ABSTRACT

Neurophysiological modifications associated to phenotypic plasticity in response to predators are largely unexplored, and there is a gap of knowledge on how the information encoded in predator cues is processed by prey sensory systems. To explore these issues, we exposed *Rana dalmatina* embryos to dragonfly chemical cues (kairomones) up to hatching. At different times after hatching (up to 40 days), we recorded morphology and anti-predator behaviour of tadpoles from control and kairomone-treated embryo groups as well as their neural olfactory responses, by recording the activity of their mitral neurons before and after exposure to a kairomone solution. Treated embryos hatched later and hatchlings were smaller than control siblings. In addition, the tadpoles from the treated group showed a stronger anti-predator response than controls at 10 days (but not at 30 days) post-hatching, though the intensity of the contextual response to the kairomone stimulus did not differ between the two groups. Baseline neuronal activity at 30 days post-hatching, as assessed by the frequency of spontaneous excitatory postsynaptic events and by the firing rate of mitral cells, was higher among tadpoles from the treated versus the control embryo groups. At the same time, neuronal activity showed a stronger increase among tadpoles from the treated versus the control group after a local kairomone perfusion. Hence, a different contextual plasticity between treatments at the neuronal level was not mirrored by the anti-predator behavioural response. In conclusion, our experiments demonstrate ontogenetic plasticity in tadpole neuronal activity after embryonic exposure to predator cues, corroborating the evidence that early-life experience contributes to shaping the phenotype at later life stages.

KEY WORDS: Behavioural plasticity, Defensive behaviour, Kairomone, Mitral neurons, Neuronal plasticity, Olfactory sensory system, Phenotypic plasticity

INTRODUCTION

Phenotypic plasticity is the ability of a single genotype to modify its phenotype (physiology, morphology and behaviour) in order to track environmental changes (West-Eberhard, 1989). Thus, phenotypic plasticity can improve fitness, despite its inherent tradeoffs (Scheiner, 1993; DeWitt, 1998; Auld et al., 2010). The concept was first applied to morphological traits (Woltereck, 1909), but virtually any trait can show plasticity in response to environmental variation (Pigliucci, 2001). However, though all types of plasticity represent (or result from) altered physiology, the proximate mechanisms involved are still poorly known (Whitman and Agrawal, 2009; Ferrari et al., 2010; Forsman, 2015). Phenotypic plasticity can be described as a chain process where sensory systems are the first step by which environmental information is acquired by the organism, and the phenotypic modification is the final product (DeWitt and Scheiner, 2004). To help clarify how plasticity has evolved, we need to know both the mechanisms producing the plastic phenotype and the selective pressures driving the evolution of such a phenotype.

Predator–prey interactions have proven very useful in understanding the ecology and evolution of phenotypically plastic traits (Benard, 2004; Ferrari et al., 2010; Warkentin, 2011). In fact, predator–prey interactions can be viewed as an arms race of sensory systems where both actors are each rewarded when they gain an information advantage over the other. Early detection is the key to achieving such an advantage, which can translate into fitness benefits (Lima and Dill, 1990; Ferrari et al., 2010). In aquatic ecosystems, where information between conspecifics and other species is mostly shared via chemical cues, olfaction is the dominant sensory system and all major groups of aquatic organisms, from protists to amphibians, display defensive behaviours upon detection of predator odour (kairomone) or alarm cues released by damaged and consumed prey (Laurila et al., 1997; Wisenden, 2003; Lass et al., 2005). These chemicals often invoke immediate behavioural responses (and, if prolonged in time, morphological and life-history trait modifications) functioning to reduce the chances of predation (Ferrari et al., 2010).

The literature on kairomone and alarm cue effects in aquatic ecosystems is extensive and focused on molluscs, crustaceans, fishes and amphibian larvae (see Ferrari et al., 2010, and references therein). In particular, tadpoles show extreme sensitivity to many different environmental stimuli during their development, and phenotypic effects induced by predator cues can be conspicuous. It is well known that developing embryos and tadpoles modify the timing of hatching/metamorphosis as well as morphology and behaviour in response to the perception of chemical cues of predation (Van Buskirk, 2001; Laurila et al., 2002; Orizaola and Braña, 2004; Ireland et al., 2007; Ferrari and Chivers, 2009; Ferrari et al., 2010), and this plasticity may result in increased survival (Mathis et al., 2008). In contrast, the neurophysiological changes underlying phenotypic behavioural plasticity induced by predator cues remain largely unexplored (Orr et al., 2007; Whitaker et al., 2011). Predator odours processed through the olfactory neural system may, for example, modulate the hypothalamo-pituitary–adrenocortical axis and hence corticosteroid production, which in turn mediates life-history, morphological and behavioural changes in tadpoles (Denver, 2009; Maher et al., 2013).

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List of abbreviations

GC	granule cell
LMM	linear mixed model
MC	mitral cell
OB	olfactory bulb
OE	olfactory epithelium
OP	olfactory pit
ORN	olfactory receptor neuron
sEPSC	spontaneous excitatory postsynaptic current

However, whether and how kairomones alter the olfactory bulb's (OB) neuronal activity remains unknown. The OB is a phylogenetically conserved cortical structure with a multi-layered cellular architecture (Ramon y Cajal, 1894); in tadpoles it includes two main neuron classes: the mitral cells (MCs) and the local interneurons (granule cells, GCs; Manzini et al., 2003; Nezlin and Schild, 2000), which can be distinguished on the basis of their distance from the surface of the bulb and their electrophysiological properties (resting potential, input resistance and firing pattern). Morphology, projections and synaptic interactions of MCs have been extensively described in rodents (Shipley and Ennis, 1996), but little is known about how MCs in amphibians and their larvae integrate electrical inputs evoked by predators' chemical signals.

To bridge these gaps, we ran a series of experiments using clutches of a frog species, the agile frog (*Rana dalmatina* Bonaparte 1840). We exposed embryos to the kairomone of larval predators (dragonfly larvae, *Anax imperator*) for 9 days and analysed its effects on key life-history traits (time of hatching, developmental stage, hatchling size) and post-hatching anti-predator behaviour before (ontogenetic behavioural plasticity) and after (contextual behavioural plasticity) a postnatal kairomone exposure (see Stamps, 2015, for plasticity terminology). Neurophysiological responses 30 days after hatching were analysed by *in vivo* whole-cell recording of MC activity from control tadpoles and tadpoles exposed to the kairomone at the embryo stage (hereafter, 'treated tadpoles') before (ontogenetic neuronal plasticity) and after (contextual neuronal plasticity) a postnatal kairomone exposure.

We predicted that embryonic exposure to the kairomone of a larval predator would induce an adaptive ontogenetic plastic response in hatchlings (delayed hatching and morphological changes; Moore et al., 1996), ultimately functioning to enhance tadpole survival. As larval anurans typically reduce their activity level when exposed to predator cues ('freezing behaviour'; Skelly, 1994; Mathis et al., 2003; Ferrari and Chivers, 2009), we predicted that treated tadpoles would show a lower baseline post-hatching activity than controls. In addition, we expected a differential contextual behavioural response according to embryonic experience, i.e. a stronger decrease of activity among treated tadpoles compared with controls when postnatally exposed to the same kairomone. Finally, we expected that the neuronal activity of MCs (the main output neurons of the OB; Czesnik et al., 2003; Davison and Katz, 2007) would be modified by embryonic kairomone exposure, as MCs are directly connected to different nuclei involved in defensive responses (Herrick, 1921). Similarly to the behavioural response, we expected a differential contextual neuronal response according to embryonic treatment.

MATERIALS AND METHODS

Model species, animal collection and housing

This study was carried out with permission from the Italian Ministry of Environment (Prot. 0035817/PNM, validity 2013–2015) and the Italian Ministry of Health (D.M. no 68/97-A, permanent validity, to the Physiology Lab, Department of Biology and Biotechnology, University of Pavia). The study was conducted in conformity with the Italian current laws for amphibian collection and detention and adhering to the Animal Behaviour Society Guidelines for the Use of Animals in Research.

The agile frog spawns in small ponds and ephemeral pools in early spring. Females leave ponds soon after laying up to 2000 eggs in a single egg mass, and only 18% of broods show multiple paternity (Lodé and Lesbarrères, 2004). Eggs are vulnerable to predation by birds, fish, newts and leeches, while larvae are subjected to a wider range of predators: dragonfly larvae, water beetles and bugs, crayfish, fish, snakes and birds; tadpoles of this species are known to show specific phenotypic responses (either morphological or behavioural) to different aquatic predators (Teplitsky et al., 2005). *Anax imperator* larvae are sit-and-wait predators, which require movements, detected either visually or via mechanosensory hairs, in order to elicit a predatory response, as immobile or dead tadpoles do not trigger predatory strikes (Skelly, 1994); dragonflies are important predators of amphibian larvae, but they do not consume amphibian eggs (P.G. and A.G., personal observation).

On 1 March 2013, we collected 10 freshly laid frog clutches from a natural, ephemeral pool near Pavia (Po Plain, Northern Italy). At the same time, 20 late-instar dragonfly larvae were collected in a different stable pond within the University Campus (Pavia, Northern Italy). We can reasonably exclude predator effects on embryos before clutch collection, because of both the ephemeral nature of the original pool, limiting dragonfly colonization, and the water temperature at collection time (+4°C), inhibiting dragonfly motility and feeding activity, and hence kairomone spreading.

We kept all animals in an unheated room with open windows under natural light conditions and mean water temperatures between 8°C (March) and 25°C (May). Agile frog clutches were individually held in opaque plastic tanks (60×60×80 cm) containing 150 l of aged tapwater and equipped with aerators. After the tadpoles hatched, we fed them *ad libitum* with rabbit chow and changed 50% of the water every other day. *Anax imperator* larvae were held individually in 250 ml plastic cups (complete water change every other day) and were fed with living freshwater amphipod shrimps *Gammarus* sp. every other day. At the end of the experiments (mid-May), all survivors (95% tadpoles, 100% dragonflies) were returned to their original sites.

Effects of embryonic kairomone exposure on tadpole life-history traits

On the day of clutch collection, we gently removed ca. 100 eggs from each clutch ball and split them into two samples of ca. 50 eggs (each consisting of a single mass with the egg jelly intact), which were placed in matched plastic tanks (30×20×20 cm, 20 tanks in total for 1000 eggs) containing 8 l of aged well water and equipped with aerators. Embryos were at Gosner developmental stage 12–18 (Gosner, 1960), and treatment began immediately.

In our split-brood design, 10 half-clutches served as a control (infusion of 50 ml of well water with no predator cues), while the other 10 half-clutches were subjected every day to infusion by a syringe of 50 ml of water containing chemical stimuli (kairomone) from three different *A. imperator* larvae fed with *Gammarus* sp. shrimps since the time of collection, but kept fasting for 48 h before infusion. Both treatments (well water and kairomone) were stopped in all tanks when the first egg in the kairomone tank hatched, which occurred 9 days after the onset of the experiment. During the treatment period, water temperature ranged from 5 to 10°C, and random temperature checks revealed only a 0.3°C mean daily difference among the 20 tanks.

Hatching time of each half-clutch was defined as when 50% of the embryos were completely detached from the yolk sac and remained immobile on the substrate surface, and was calculated as the time (hours) since the start of the treatment. Hatchling developmental stage was determined according to Gosner (1960). Immediately after hatching (stage Gosner 23–24) and 40 days later (stage Gosner 34, range 30–37), 5 tadpoles/tank were collected and preserved in 10% formalin to measure mass and morphological traits.

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Tadpoles were weighed three times (Sartorius R200D balance, Göttingen, Germany; accuracy 0.01 mg) and photographed (three times) in lateral view within a small glass chamber under standardized conditions (light, exposure and distance of the subject set constant for all pictures) by a digital camera (Panasonic Lumix DMC FZ28, Kadoma, Osaka, Japan; 10.1 megapixel sensor resolution, 3.648×2.736 pixels output images). Pictures were processed with ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA) to determine developmental stage and measure total length, body and tail length, maximum body thickness, maximum tail fin thickness, maximum tail muscle thickness, and eye size at hatching and close to metamorphosis. Measurements were taken by the same observer (A.G.), who was blind to embryonic treatment. Repeatability of these measurements calculated on a subsample of 20 tadpoles was very high (r_i or intra-class correlation coefficient varying from 0.94 to 0.99, with F-values ranging from 48.9 to 4439, all $P<0.0001$; Measey et al., 2003). Ratios between tail length and body length, between tail thickness and tail length, between body thickness and body length and between tail muscle thickness and tail fin thickness were also calculated.

Effects of embryonic and postnatal kairomone exposure on tadpole anti-predator behaviour

We conducted 10 min trials in experimental tubs (15×10×10 cm) using matched pairs of tadpoles from the treated and control groups from each clutch of origin (hereafter 'strain'), 10 days after hatching (stage 25–26 Gosner), to assess the activity of the larvae before and after kairomone infusion (20 pairs, 40 individuals in total). Pairs of treated and control tadpoles from each strain were inserted in two transparent, adjacent tubs, filled with 250 ml of aged well water, and left to acclimatize for 15 min. The tubs were visually isolated from each other by a cardboard barrier. The trials consisted of a 5 min pre-stimulus recording period (before infusion), a 30 s infusion period (water with kairomone) and a 5 min post-stimulus recording period (after infusion). Behaviour (activity) of the tadpoles was recorded, and a decrease in activity was considered a fright response (Petranka and Hayes, 1998). To measure activity, we drew two perpendicular lines across the centre on the outer bottom of the tub and counted the number of line crosses during the two observation periods. We considered that a tadpole crossed a line when its entire body was on the other side of the line. During the infusion period, 5 ml of water containing kairomones from three different *A. imperator* larvae kept fasting for 48 h before trials was emptied slowly by a syringe on the side of the cup to minimize disturbance. In order to rule out that any contextual effect of kairomone infusion on motility was simply due to the mechanical disturbance induced by syringe infusion, we carried out control trials on a sample of tadpoles that had not been treated with kairomone as embryos (N=20). These tadpoles were exposed to a 5 ml well water infusion by a syringe (i.e. without kairomone) at 10 days of age, and their activity did not significantly differ before and after the infusion (movements before infusion: 5.55±1.69; after infusion: 4.25±1.44, means± s.e.m., paired samples t-test on $\log_{10}$-transformed activity data: $t_{39}=0.50$, $P=0.62$). The trials were performed indoors and tadpoles were video recorded over the whole trial (JVC GZ-MG140E digital video camera, Milan, Italy). Videos for three kairomone trials at 10 days of age failed and were discarded, thus reducing the sample size to 34 tadpoles. Each tadpole was tested once and then discarded from the next behavioural trials.

We replicated the previous test using pairs of treated and control tadpoles 30 days after hatching (mean stage 29 Gosner, range 28–31, 30 pairs, 60 individuals in total) from each strain, in order to test long-term behavioural differences between treated tadpoles and their control siblings. All trials were video-recorded for 5 min both before and after the infusion period.

Effects of embryonic and postnatal kairomone exposure on MC activity of tadpoles

Thirty days after the tadpoles hatched (an age that permits neurophysiological tests on tadpoles to be done), concomitantly to the second behavioural test, we measured, by patch-clamp recordings, the neurophysiological response in MCs of both control and treated tadpoles. We first recorded, for both groups, baseline neuronal activity through perfusion of a bath solution (see below for details). Then, we puffed a solution composed of bath solution mixed with dragonfly kairomone (concentrated 10 mmol l^{-1}) in front of the olfactory pit (OP) to detect the effect of predator chemical cues on MC activity of both groups. We used the minimum sample sizes to obtain statistically meaningful data, while keeping the number of animals that were euthanized to a minimum. In total, we used 13 control and 16 treated tadpoles (mean stage 29 Gosner, range 27–32) from different strains, where sample size varied according to each experiment performed (see Results), as some neurons exited the range of accepted stability parameters (series resistance) during the recording (see below for further details).

In vivo preparation for patch-clamp recordings

Tadpoles were anaesthetized in a mixture of ice and water for dissection. A custom-built harp was placed over the tadpole and the preparation was fixed in place in the recording chamber with 3% low-melting point agarose, slice anchor (Warner Instruments, Hamden, CT, USA) and insect pins (Sigma, Milan, Italy). The skin of the head was cut and the brain was opened along the midline of the recording. The ending part of the tail was also cut to reduce animal movements during experiments. For recording, the tadpole was held submerged in a custom-shaped Sylgard chamber with slice anchor and insect pins and constantly perfused with a fresh external bath solution (see below); all experiments were performed at room temperature. Heart and breath rhythm could be observed in healthy animals and were monitored through the experiments. The brain was then viewed using Nomarski optics (Olympus BX51WI, Shinjuku, Tokyo, Japan).

The preparation was allowed to stabilize for 15 min, and MC *in vivo* whole-cell recordings were obtained from the OB using the blind patch-clamp method (Blanton et al., 1989). Because it is impossible to visually identify the mitral cells (patched neurons) by this method, we measured some electrophysiological parameters of cells such as the resting potential, the input resistance and the firing pattern, which allowed us to distinguish the MCs from the GCs.

Patch electrodes with a tip diameter of 1–2 µm and approximately 7–10 MΩ resistance were fabricated from borosilicate glass with a 1.8 mm outer diameter (Hilgenberg, Malsfeld, Germany) using a two-stage electrode puller (Narishige, Tokyo, Japan) and fire polished. The patch pipette was filled with an intracellular solution (see below). Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in voltage-clamp mode at a holding potential of −70 mV, while cells firing at resting potential were recorded in current-clamp mode. A 200B amplifier (Axon Instruments, Biberach an der Riss, Germany) was interfaced to pClamp command/record software through a Digidata 1440A analog/digital converter (Molecular Devices, Biberach an der Riss, Germany; low-pass filter 10 kHz, sampling rate 100 kHz). Voltage pulses were delivered from a microcontroller to a D/A converter and then to the patch-clamp amplifier to assess the impedance in the whole-cell configuration. Series resistance was monitored by measuring passive current transients induced by −10 mV hyperpolarizing voltage steps from a holding potential of −70 mV as previously described (D'Angelo et al., 1993; Brandalise et al., 2012). Accepted deviations for this parameter in transient currents were less than 15%. The data were digitalized off-line using an 8-pole Bessel filter, an A/D converter and a PC. Experimental data were analysed using pClamp (Molecular Devices) and Origin (Microcal Software, Northampton, MA, USA) software.

Solutions used in neurophysiological trials

The composition of the bath solution was (mmol l^{-1}): 135 NaCl, 2 KCl, 3 $CaCl_2$, 1.5 $MgCl_2$, 10 glucose, 10 Hepes, pH 7.3, 255–260 Osm. The pipette solution used for whole-cell recording contained (mmol l^{-1}): 5 NaCl, 47 KCl, 1.5 $MgCl_2$, 120 potassium gluconate, 20 Hepes, 1 EGTA, 2 Na_2-ATP and 0.3 Na_2-GTP. All chemicals were purchased from Sigma/Fluka (Milan, Italy).

The kairomones (collected with the same procedure used for postnatal behavioural trials) were first dissolved in bath solution (10 mmol l^{-1} stock) and then puffed locally in front of the OP. A fast perfusion system was located close to the puffer pipette in order to have a constant, anterior-to-posterior flux of bath solution which rapidly washes the puffed kairomones. In this way the applied kairomones can exert a local action on the olfactory receptor neurons, but thanks to the fast removal from the extracellular space, we avoided a possible significant spill-over in the proximity of the recorded mitral cells.

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Short pressure pulses (2.7 kPa, 1.5 s) were delivered by a Picospritzer (PDES-2L NPI Electronic Instruments, Tamm, Germany) and were made in order to eject a small and local amount of kairomone solution. Patch pipettes of 3–4 MΩ were loaded with solution containing kairomones, and this was puffed 3–4 times per experiment with intervals of at least 3 s between one puff and the other. Recordings for both the spontaneous activity and the firing frequency were made from 5 min before kairomone application until 3–4 min after the last puff application to record the neuronal activity and also activity when kairomone solution was washed out.

Statistical analyses

We first ran linear mixed models (LMMs) (Zuur et al., 2009) to investigate differences in hatching time between treated and control clutches, using embryonic treatment (well water or kairomone) as a fixed factor, and stage at collection of each clutch as a covariate. Clutch of origin (strain) was included as a random intercept effect.

The effects of embryonic kairomone exposure on embryo development (Gosner stage) and morphology at hatching were analysed by means of LMMs, with embryonic treatment as a fixed factor and strain as a random intercept effect. We dismissed developmental stage at collection as a covariate because the factor 'strain' fully captured the variance due to clutch age. We explored the existence of genotype by environment interactions by testing, for each trait, whether treatment effects significantly varied among strains. This was achieved by including a by-strain random slope for the kairomone treatment effect in linear mixed models of morphological traits. Significance of random slope effects and of fixed effects was tested by means of likelihood ratio tests, by computing the difference in −2 log-likelihood of the model including and the one excluding only the model term of interest, which is χ^2 distributed. Similar analyses were run to investigate the effects of embryonic kairomone treatment on tadpole development (Gosner stage) and morphology 40 days after hatching.

LMMs were used to compare activity ($\log_{10}$-transformed values to improve normality) of treated and control tadpoles before and after infusion of water containing kairomone (postnatal kairomone treatment) at 10 and 30 days after hatching. We used tadpole movements as the response variable, embryonic kairomone treatment and postnatal kairomone treatment as fixed factors, strain and tadpole identity as random intercept effects, and time of day and Gosner stage as covariates. A similar model was run on pooled data from both age groups, but in this case we included age (day 10 or 30) as a fixed effect instead of Gosner stage. Interaction terms between fixed factors were included in the models.

Finally, LMMs were performed to compare various electrophysiological parameters recorded in MCs according to embryonic kairomone treatment, accounting for strain identity as a random intercept effect. For measures recorded both before and after a kairomone perfusion on the same MCs (sEPSCs and firing frequency), models were run by including strain and cell identity as random intercept effects. The reported N values refer to the number of cells analysed (one cell for each tadpole).

LMMs were fitted using the lme4 library (v1.0-5) of R 3.0.2 (R Core Development Team, 2013) and SAS 9.3 Proc Mixed. Degrees of freedom were estimated according to the Kenward–Roger method. Parameter estimates and mean values are reported with their associated s.e.m.

RESULTS

Embryonic kairomone exposure modifies life-history traits and post-hatching behaviour of tadpoles

Hatching time was significantly affected by embryonic kairomone exposure ($F_{1,9}$=33.96, P=0.0003) and by embryo developmental

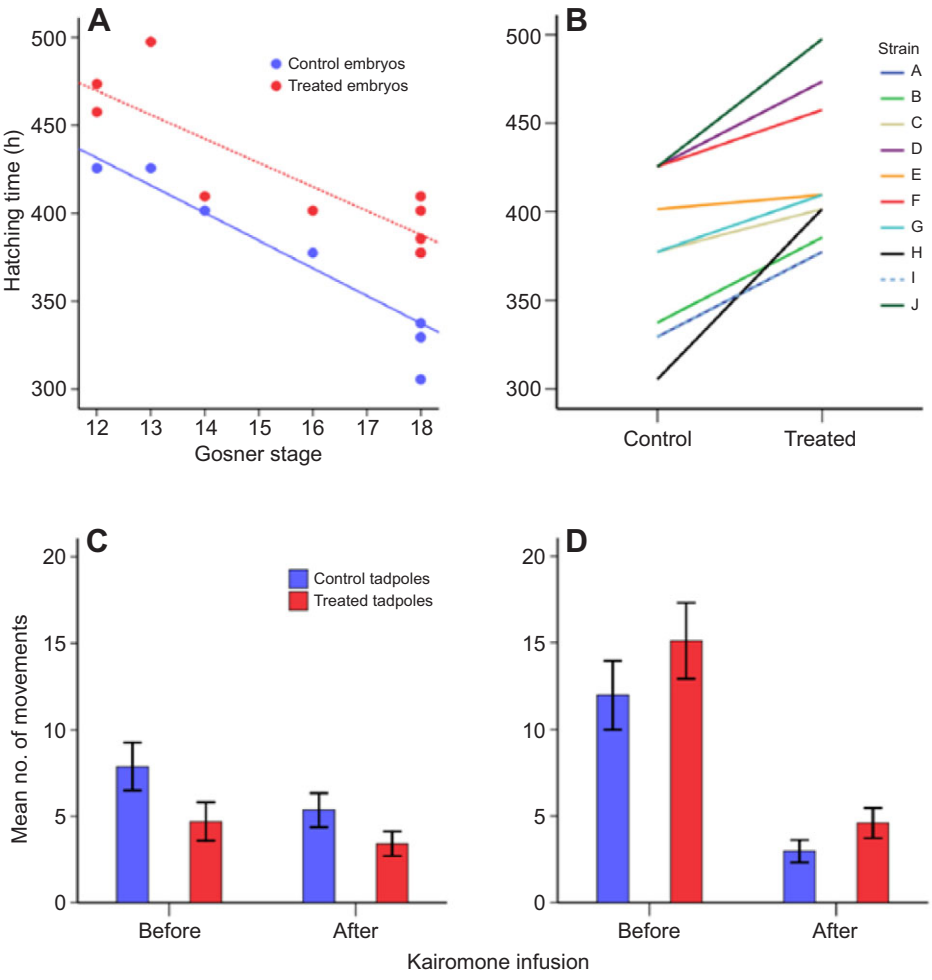

Fig. 1. Embryonic exposure to kairomone affects embryo and tadpole phenotypic traits. (A) Hatching time (when 50% of eggs in each half-clutch hatched) of control (well water) and kairomone-treated embryos according to their developmental stage at collection time (P<0.001). R^2 of regression lines was 0.84 for control and 0.75 for kairomone-treated embryos. (B) Hatching time of control and treated embryos according to strain (i.e. genotype). (C) Mean activity of tadpoles from control and treated embryo groups at 10 days before and after kairomone infusion (see Table 2 for statistics). (D) Mean activity in the same experimental set-up as for C at 30 days. Error bars represent s.e.m. (see Table 2 for statistics).

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Table 1. Developmental stage and morphology of tadpoles exposed during the embryonic phase to well water (control) or kairomone (treated)

Variables	Control	Treated	Estimate	χ^2	*P*	$P_{G\times E}$
Hatching						
Stage (Gosner)	23.78±0.07	23.61±0.08	−0.17±0.14	1.48	0.22	0.06
Total length (mm)	11.29±0.06	10.80±0.11	−0.48±0.14	8.35	0.004	0.0002
Body length (mm)	3.96±0.02	3.79±0.04	−0.17±0.07	5.34	0.021	0.0003
Mass (mg)	10.26±0.22	9.23±0.26	−1.03±0.54	3.35	0.07	0.0009
Tail length (mm)	7.33±0.06	7.02±0.09	−0.31±0.08	9.31	0.002	0.06
Tail depth (mm)	2.35±0.03	2.15±0.05	−0.19±0.07	5.70	0.017	0.0001
Tail length:body length	1.86±0.02	1.86±0.02	−0.01±0.01	0.01	0.97	0.36
Tail depth:length	0.32±0.01	0.31±0.01	−0.01±0.01	2.57	0.11	0.0002
Day 40						
Stage (Gosner)	34.53±0.22	32.79±0.25	−1.81±0.47	24.78	<0.0001	0.54
Total length (mm)	33.81±0.40	31.74±0.45	−0.21±0.07	7.19	0.007	0.71
Body length (mm)	11.68±0.12	10.80±0.14	−0.88±0.25	8.48	0.004	0.33
Body depth (mm)	6.66±0.09	6.11±0.10	−0.47±0.16	6.43	0.011	0.18
Body depth:length	0.56±0.01	0.57±0.01	0.01±0.01	0.18	0.67	0.99
Mass (mg)	344.80±10.4	276.70±10.8	−68.0±22.2	7.15	0.008	0.07
Tail length (mm)	22.14±0.31	20.93±0.32	−1.20±0.45	5.92	0.015	0.85
Tail depth (mm)	7.39±0.11	6.65±0.12	−0.74±0.19	10.01	0.002	0.25
Tail length:body length	1.89±0.02	1.94±0.02	0.04±0.02	3.50	0.06	0.87
Tail depth:length	0.33±0.01	0.32±0.01	−0.02±0.01	9.69	0.002	0.39
Muscle depth (mm)	2.91±0.04	2.64±0.04	−0.27±0.06	10.51	0.001	0.15
Eye size (mm^2)	5.67±0.22	4.51±0.22	−1.15±0.34	8.23	0.004	0.34

Control and treated values represent means±s.e.m. Estimated treatment effects (slope and s.e.m.) from linear mixed models are also shown, with corresponding *P*-values from likelihood ratio tests. $P_{G\times E}$ corresponds to the *P*-value of a likelihood ratio test of the genotype by environment interaction (by-strain random slope for treatment effect; see 'Statistical analyses' in Materials and methods). Sample size is 50 individuals per treatment for all traits (5 individuals×10 half-clutches).

stage at collection ($F_{1,8}$=50.16, *P*=0.0001). As expected, late-staged clutches hatched earlier than early-staged clutches (estimate: −14.66±2.07 h Gosner stage $unit^{-1}$; Fig. 1A), but treated embryos always hatched later than their control siblings (mean hatching time: 419.1±14.4 versus 373.5±13.3 h, ca. 2 days difference; Fig. 1A). Most strains exhibited a similar response to environment (Fig. 1B).

Developmental stage at hatching did not differ between control and treated siblings or among strains (Table 1). Thus, kairomone-treated embryos developed more slowly and hatchlings were also significantly smaller than their control siblings in all morphological measures but body mass (Table 1). The ontogenetic and morphological effects of embryonic kairomone exposure were persistent, as similar differences in body traits between control and exposed siblings were still detectable at 40 days of age, when treated tadpoles also showed a significantly lower developmental stage compared with controls (Table 1). At hatching, we detected statistically significant genotype by environment interactions in most body traits, which, however, disappeared at 40 days of age (Table 1). Close scrutiny revealed that such genotype by environment effects on body traits at hatching were due to a single strain (strain J) showing a stronger response to embryonic kairomone treatment than the other strains.

Tadpole activity at 10 days of age significantly differed according to both embryonic treatment and postnatal kairomone exposure (Fig. 1C, Table 2), but the effect of postnatal kairomone exposure did not significantly vary according to embryonic treatment (no significant embryonic×postnatal treatment interaction, Table 2). In this model, we accounted for the confounding effects of time of day (daily tadpole activity regularly decreased from morning to evening) and developmental stage (Table 2). Treated tadpoles exhibited lower motility than their control siblings both before and after kairomone infusion, which had, however, a significant suppressive effect on the activity of both groups (Fig. 1C, Table 2). Similarly, at day 30, activity dramatically decreased following kairomone infusion, while the effect of the embryonic kairomone exposure disappeared, both tadpole groups showing high baseline motility (Fig. 1D, Table 2). Again, there was no significant embryonic×postnatal treatment interaction (Table 2). The global model run on pooled data from both age groups showed that the overall effect of embryonic kairomone exposure on tadpole movements significantly differed between day 10 and day 30 (age×embryonic treatment interaction in Table 2), corroborating the results of the tests run separately on each age group (Table 2). In addition, the effect of postnatal kairomone infusion significantly differed between day 10 and day 30 (age×postnatal treatment interaction in Table 2), with movements after kairomone infusion decreasing more markedly at day 30 than at day 10 (Fig. 1C,D).

Table 2. Mixed models of tadpole activity (movements) in relation to embryonic kairomone treatment and postnatal kairomone exposure

Variables	*F*	d.f.	*P*
Day 10			
Embryonic treatment	5.25	1,22	0.032
Postnatal treatment	7.38	1,32	0.010
Embryonic×postnatal treatment	0.49	1,32	0.49
Time of day (h)	4.22	1,29	0.049
Gosner stage	2.65	1,21	0.12
Day 30			
Embryonic treatment	2.60	1,47	0.11
Postnatal treatment	74.18	1,58	<0.0001
Embryonic×postnatal treatment	0.13	1,58	0.72
Time of day (h)	0.21	1,29	0.65
Gosner stage	1.29	1,55	0.26
Global model			
Age	1.74	1,88	0.19
Embryonic treatment	0.05	1,80	0.83
Postnatal treatment	54.23	1,90	<0.0001
Age×embryonic treatment	8.31	1,80	0.005
Age×postnatal treatment	19.18	1,90	<0.0001
Embryonic×postnatal treatment	0.02	1,90	0.89
Age×embryonic×postnatal treatment	0.41	1,90	0.53
Time of day (h)	0.51	1,88	0.48

Strain and individual identity were included as random intercept effects. Sample sizes were 34 tadpoles at day 10 and 60 tadpoles at day 30.

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In short, ontogenetic behavioural effects of embryonic kairomone exposure were detectable within the first 10 days from hatching, diminishing by day 30, while the intensity of the contextual behavioural response to postnatal kairomone exposure increased with age in both tadpole groups.

Embryonic kairomone exposure induces variation in MC voltage-dependent currents during *in vivo* whole-cell recording experiments

An image of a typical *in vivo* recording preparation is shown in Fig. 2A, where the olfactory pit (OP), the olfactory epithelium (OE), the OB and the telencephalon can be seen. MCs were located below the GCs, in a single lamina, the MC layer, with their dendrites spanning the OB, and showed more depolarized resting potentials compared with GCs (−53.2±1.12 mV, N=13 versus −66.5± 1.55 mV, N=4; mixed model, $F_{1,15}$=35.86, P<0.0001; Fig. 2B,C) in agreement with previous studies (Chen and Shepherd, 1997; Heyward et al., 2001; Scheidweiler et al., 2001; Arruda et al., 2013). In MCs, action potentials evoked by threshold current injections occurred with a long delay of up to 300 ms (N=13, Fig. 2B). Moreover, a voltage-clamp analysis of passive membrane properties obtained by −10 mV hyperpolarizing voltage steps from a holding potential of −70 mV (see '*In vivo* preparation for patch-clamp recordings' in Materials and methods for further details)

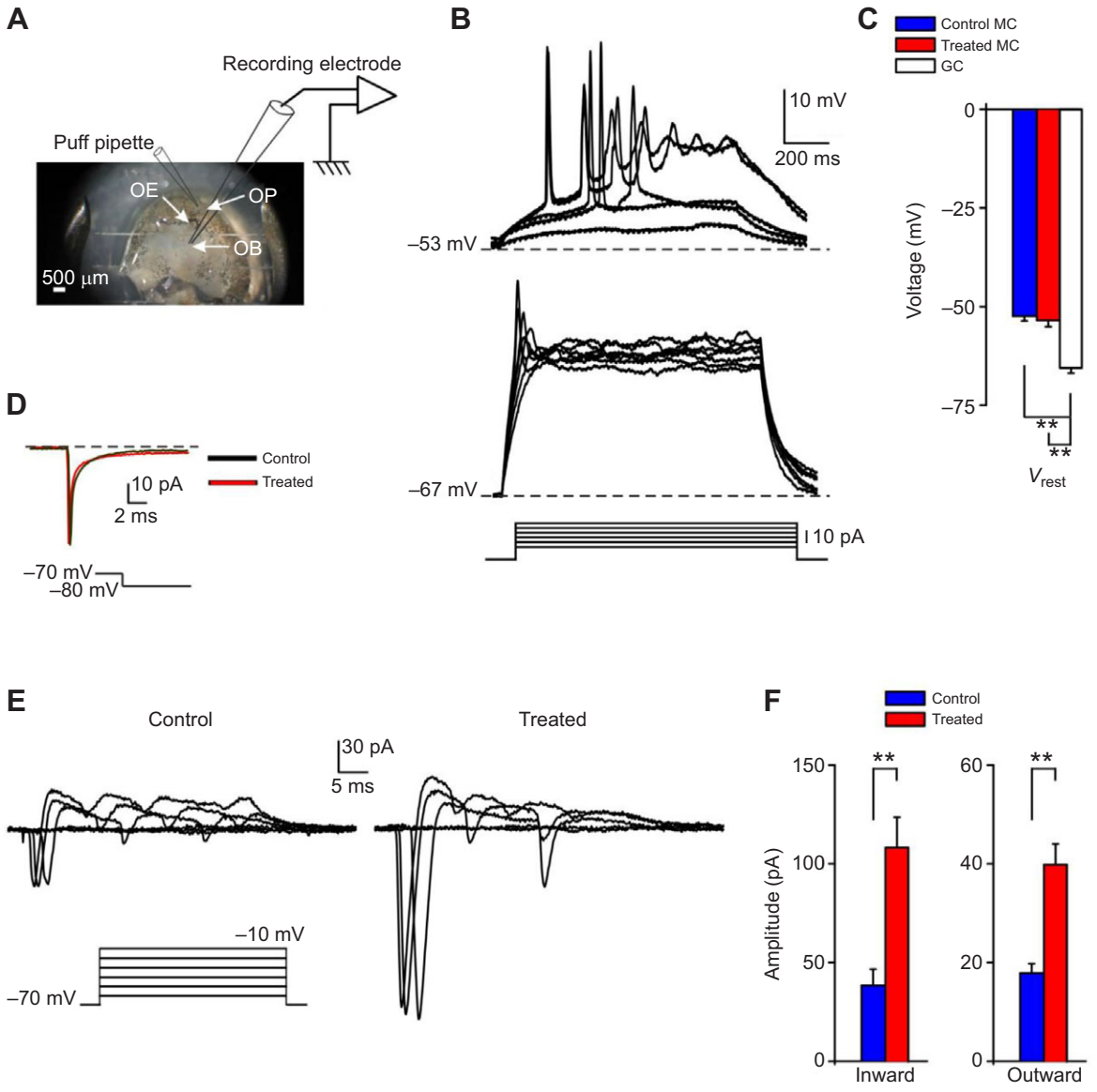

Fig. 2. Recording set-up and electrophysiological characterization of tadpole mitral cells. (A) The head of a tadpole immobilized in a recording chamber with the olfactory epithelium (OE) and the olfactory bulb (OB) exposed for patch-clamp recording. The puff pipette, filled with kairomone solution, is placed in front of the olfactory pit (OP). (B) A typical firing response of a mitral cell (MC) to depolarizing steps of increasing amplitude. Note the delay in the occurrence of the first spike, especially with low current injections, compared with a granule cell (GC) response. (C) Mean resting potential (V_{rest}) for MCs of tadpoles from control and treated embryo groups (hereafter control and treated tadpoles), and for GCs. Error bars represent s.e.m. with asterisks showing statistically significant differences (MCs versus GCs: P<0.0001). (D) Transient currents elicited by –10 mV voltage steps from a holding potential of –70 mV were induced to analyse passive properties and to assess the stability of the patch at the beginning and at the end of the recording; no significant difference was detected between MCs of control and treated tadpoles. (E) Depolarizing voltage steps from a holding potential of –70 mV to –10 mV were delivered to assess active membrane currents in MCs from control and treated tadpoles. (F) Mean amplitude of inward and outward currents for MCs from control versus treated tadpoles. Error bars represent s.e.m. with asterisks showing statistically significant differences (P<0.001 for both inward and outward currents).

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showed that MCs of control tadpoles had an input resistance of 184.7±17.7 MΩ (N=13), which is significantly lower than that of GCs (315.5±7.9 MΩ, N=4; mixed model, $F_{1,14}$=9.13, P=0.009), according to previous studies (Chen and Shepherd, 1997; Scheidweiler et al., 2001; Arruda et al., 2013; Heinbockel et al., 2004).

The resting potential of MCs did not differ between control and treated tadpoles (respectively, −53.2±1.12 mV, N=13 and −54.2±0.03 mV, N=13; mixed model, $F_{1,24}$=0.43, P=0.52; Fig. 2C). Similarly, the input resistance of MCs of treated tadpoles did not exhibit a significant change compared with controls (210.7±13.7 MΩ, N=16; mixed model, $F_{1,26}$=1.20, P=0.28; Fig. 2D).

In contrast, responses to +10 mV depolarizing voltage steps from −70 mV to −10 mV revealed an inward rectifier current and a deactivating outward current in MCs (Fig. 2E). The initial inward current was significantly smaller in control tadpoles (37.6±5.6 pA, N=13) than in treated ones (107±13.9 pA, N=16; mixed model, $F_{1,25}$=16.97, P=0.0004; Fig. 2F). The outward current was also significantly greater in amplitude in treated tadpoles compared with controls (40.2±4.6 pA, N=16 versus 18±1.1 pA, N=13; mixed model, $F_{1,27}$=17.70, P=0.0003, Fig. 2F).

Thus, embryonic kairomone exposure did not modify the passive electrophysiological properties of MCs, but affected their active properties.

Embryonic and postnatal kairomone exposure elicits an increase in the frequency of spontaneous activity of MCs

When perfused with bath solution as a control, MCs of the treated tadpoles showed a significantly higher baseline frequency of sEPSCs compared with control MCs (4.84±0.28 Hz, N=16 versus 1.47±0.09 Hz, N=13; Fig. 3A,B, Table 3). When bath solution with dragonfly kairomone was puffed close to the OP during the recording section (see Materials and methods), the frequency of spontaneous activity significantly increased in both groups (control cells: 2.4±0.11 Hz, N=11; treated cells: 7.8±0.24 Hz, N=15; Fig. 3C, Table 3), but this increment was much higher for treated MCs than for the control group (embryonic×postnatal treatment interaction in Table 3). However, both treated and control olfactory receptor neurons (ORNs) expressed kairomone-activated receptors, indicating that tadpoles possess intrinsic sensitivity to predator chemical cues.

To investigate the mechanism responsible for the increased frequency of sEPSCs among treated MCs, we performed a quantal analysis (Nusser et al., 2001; Granseth and Lindstrom, 2003; Sola et al., 2004). The higher frequency of sEPSCs among treated MCs may depend on different mechanisms, including: (1) a greater number of synaptic contacts between ORNs (presynaptic neurons) and MCs (postsynaptic neurons); and (2) an increase in the number of neurotransmitter release sites per synapse. The mean amplitude of these sEPSCs was 5 pA, determined by the peak of a Gaussian curve fitted to the size distribution (Fig. 3D,E). A few large sEPSCs could be multiples of this unitary amplitude. The histograms exhibited similar distributions in both control and treated MCs, but with a significantly higher peak (i.e. a higher frequency at which the events occur) in the latter cells (Fig. 3E). In response to puff application of bath solution with dragonfly kairomone, the Gaussian distribution was maintained, but the peaks increased for both control and treated cells (Fig. 3D,E). The fact that the frequency of events, but not the amplitude, was enhanced by kairomone perfusion was consistent with an increase in the number of synaptic contacts per MC or a possible increase in the firing frequency of the presynaptic ORNs. An increase in the number of neurotransmitter release sites per synapse would instead be associated with a Gaussian multipeak, with peaks approximately multiples of each other (Paulsen and Heggelund, 1996).

Embryonic and postnatal kairomone exposure elicit an increase in MC firing rate

MCs of the treated tadpoles showed a significantly higher baseline firing frequency than MCs of control tadpoles (0.74±0.09 Hz, N=15 versus 0.12±0.07 Hz, N=13; Table 3, Fig. 4A,C), as was expected from the difference in network activity observed in the sEPSC analysis. With local puff application of bath solution with dragonfly kairomone, firing rate increased markedly in both groups of MCs (control cells: 0.96±0.14 Hz, N=11; treated cells: 5.32±0.21 Hz, N=13; Table 3, Fig. 4A,B), but again the difference in firing rate between control and treated cells widened further (embryonic×postnatal treatment interaction in Table 3, Fig. 4C). Kairomone solution also induced frequent bursts of action potentials in the treated group (5.2±0.9 action potentials, N=12; Fig. 4B), but not in the control group (Fig. 4A). Two minutes after the last application was terminated, the kairomone solution was fully washed out, so that bursting behaviour ceased and the firing frequency returned to values not significantly different from those recorded before kairomone application (control cells: 0.25±0.04 Hz, N=5; treated cells: 1.15±0.12 Hz, N=8; Fig. 4A,B; mixed model with wash-out versus baseline firing frequency and embryonic treatment as a fixed factors, run on the subsample of cells for which we were able to record both baseline and post-wash-out firing frequency; effect of wash-out: $F_{1,17}$=3.04, P=0.10; effect of embryonic treatment: $F_{1,20}$=18.6, P=0.004; interaction: $F_{1,17}$=1.26, P=0.28).

DISCUSSION

We demonstrated that agile frog embryos exposed to larval predator cues markedly modified various phenotypic traits, including hatching time, body size and post-hatching motility, in response to the perceived risk of future predation. Remarkably, embryonic exposure to larval predator cues also altered the activity of both pre- and postsynaptic olfactory neurons: we found a significant difference in baseline sEPSCs and in firing rate between mitral neurons of control and treated tadpoles, and this difference was magnified after local puff kairomone perfusion. Thus, the contextual effect of the predator stimulus was strongly affected by the embryonic predator experience. Similarly, treated tadpoles showed lower baseline motility than control siblings at 10 days of age, but notably, at this age the two tadpole groups responded to postnatal kairomone exposure in a similar way. In addition, in behavioural trials at 30 days of age, we detected a similar baseline motility between treated and control tadpoles, which strongly decreased after postnatal kairomone exposure, irrespective of embryonic treatment.

A decrease in tadpole activity after exposure to predator cues is consistent with an adaptive anti-predator response because dragonfly larvae can most probably detect and capture actively moving prey (Skelly, 1994; Mathis et al., 2003, 2008). The contextual behavioural response of agile frog larvae to dragonfly kairomone might thus be, to a large extent, innate (Lima and Dill, 1990; Scheurer et al., 2007; Epp and Gabor, 2008), as control tadpoles displayed the typical anti-predator behaviour upon their first postnatal experience with the odour of the predator (Fig. 2C,D).

MCs of treated tadpoles were more sensitive and responsive compared with those of control siblings and, though neuronal firing frequency strongly increased in both tadpole groups after local

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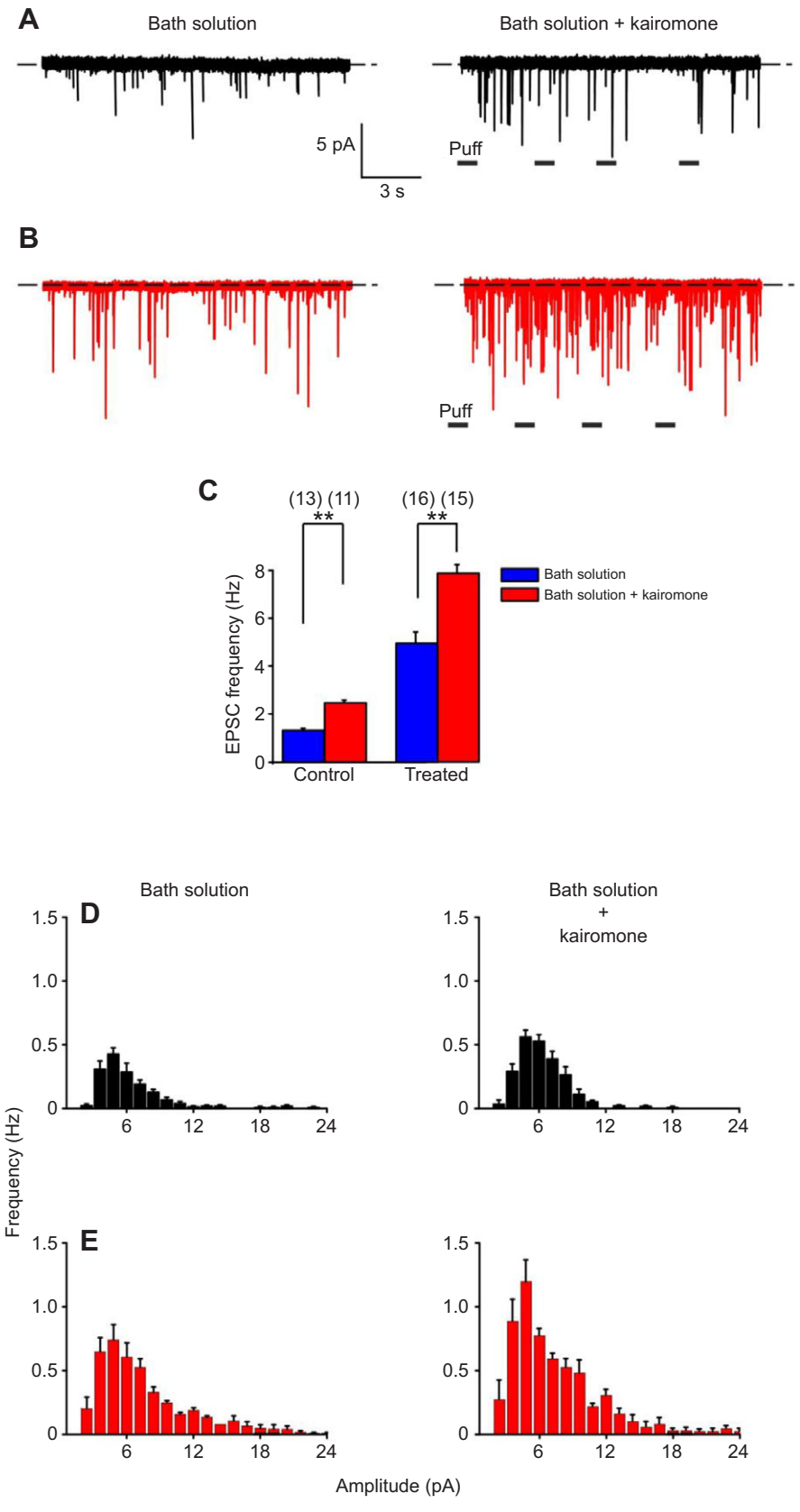

Fig. 3. Embryonic exposure to kairomone increases the amplitude and frequency of spontaneous excitatory postsynaptic currents and sensitivity of tadpole MCs to local puff of kairomone perfusion. (A) Activity of a representative MC from a control tadpole before (bath solution) and after kairomone solution puff application (bath solution with dragonfly kairomone). Note that cells from previously unexposed tadpoles also responded to kairomone solution. (B) Activity of a representative MC from a treated tadpole before (bath solution) and after kairomone solution puff application (bath solution with dragonfly kairomone); note that the spontaneous excitatory postsynaptic current (sEPSC) frequency was higher than in the cell from a control tadpole even before kairomone solution application; puff application of kairomone enhanced sEPSCs to a greater extent in treated than in control cells. (C) Mean sEPSC frequency of recorded MCs from both control and treated tadpoles in the two experimental settings (bath solution and bath solution with dragonfly kairomone). *N* values are given in parentheses; error bars represent s.e.m. with asterisks showing statistically significant differences (see Table 3 for statistics). (D) Distribution frequency of sEPSCs binned at intervals of 1 pA in control MCs before and after kairomone solution puff application; note that data could be fitted with a single Gaussian that increased in amplitude (frequency of sEPSC) when kairomone was applied. (E) Quantal analysis for the same experiments as in D, but in MCs from the treated group; again, data could be fitted with a single Gaussian that increased its amplitude (frequency of sEPSC) in response to kairomone solution.

application of kairomone, the firing pattern switched from a single spike to a robust bursting pattern (Cang and Isaacson, 2003) in neurons from treated tadpoles only. The strong increase in sEPSC frequency and in firing rate of MCs of treated tadpoles was consistent with an increase in the number of synaptic contacts between ORNs (presynaptic neurons) and MCs (postsynaptic neurons) or an increase in the firing frequency of the synaptically connected ORNs, rather than with an increase in neurotransmitter

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Table 3. Mixed models of sEPSCs and firing frequency of MCs in relation to kairomone application (postnatal treatment) and embryonic treatment

Variables	*F*	d.f.	*P*
sEPSCs			
Embryonic treatment	381.4	1,51	<0.0001
Postnatal treatment	76.43	1,51	<0.0001
Embryonic×postnatal treatment*	18.95	1,51	<0.0001
Firing frequency			
Embryonic treatment	329.48	1,27	<0.0001
Postnatal treatment	441.63	1,26	<0.0001
Embryonic×postnatal treatment‡	209.80	1,26	<0.0001

sEPSCs, spontaneous excitatory postsynaptic currents; MCs, mitral cells. Strain and individual cell identity were included as random intercept effects. **Post hoc* test; all pairwise differences statistically significant at $P<0.005$. ‡*Post hoc* test; all pairwise differences statistically significant at $P<0.001$, except the comparison between control cells after kairomone puffing and baseline treated cells ($P=0.25$).

release sites. In addition, the permanently increased baseline activity of kairomone-primed mitral neurons and their higher sensitivity/response to postnatal experiences with predator cues revealed how strong the effects of embryonic experience on the central nervous system (CNS) were, and how persistently the information obtained during early stages of development could be retained by OB neurons. In fact, MCs of treated tadpoles responded differently to kairomone application compared with those of control tadpoles up to 1 month after their exposure to the kairomone during egg development.

In short, we documented an overall decline of ontogenetic effects on anti-predator behaviour during tadpole development, while ontogenetic effects on neuronal activity apparently persisted over time. Moreover, we never observed a difference in contextual behavioural plasticity according to embryonic kairomone treatment (Dalesman et al., 2015), while the contextual neuronal response was strongly modified by prenatal predator exposure. The decline of ontogenetic behavioural effects over time and the lack of a differential contextual behavioural response to predator cues according to embryonic treatment may be due to the fact that, unlike neural activity, the defensive behaviour should be continuously adjusted to the current level of predation risk. If predator chemical cues are removed from the environment, the anti-predator behavioural response (freezing) should disappear over time (Gonzalo et al., 2009), because there would be important costs to pay in maintaining activity, and hence foraging, at low levels in the absence of predators. However, the memory of the embryonic dragonfly treatment was retained by the CNS, as revealed by the neuronal activity changes in MCs of treated tadpoles at 30 days, suggesting that behaviour was a more labile trait than neurophysiology (Dalesman et al., 2013) or morphology (Saino et al., 2003). Indeed, it remains to be elucidated whether and how the observed altered activity patterns in MCs actually translate in the inhibition of muscular fibres resulting in tadpole behavioural freezing. Admittedly, amphibians present strong synaptic connections between the olfactory bulb and the amygdala (Herrick, 1921; Moreno et al., 2005), which plays a crucial role in their fear behaviour as described for mammals (Roseboom et al., 2007).

In fact, relatively little is known about the neurophysiological processes that underlie the plastic responses of prey to their predators. A link between neurophysiological changes and the parallel behavioural effects induced by predator cue detection was demonstrated in molluscs (*Lymnea stagnalis*; Orr et al., 2007; Lukowiak et al., 2008); in this case, however, an increase of breathing and overall defensive behaviour was associated with a decrease in firing and bursting activity of a key neuron in mediating both vigilance behaviours and memory formation. In crustaceans (e.g. *Daphnia pulex*), cholinergic- and GABAergic-dependent pathways are involved in the perception and transmission of different predator cues, suggesting that the nervous system mediates the development of specific defences against a particular predator species (Weiss et al., 2012). In fish, exposure to a putative alarm substance enhances optical alertness, suggesting an action on the CNS that affects visual acuity (Pfeiffer et al., 1985). In mammals, exposure to predator odour causes behavioural inhibition (freezing), activation of the neuroendocrine stress axis and correlated changes in CNS limbic circuitry associated with fear and anxiety (Figueiredo et al., 2003; Halpern and Martínez-Marcos, 2003; Beny and Kimchi, 2014). In light of our results, we speculate that a similar mechanism can be at work in anuran embryos chronically exposed to a predator kairomone (Denver, 2009; Maher et al., 2013). Recent findings show that stress hormones play a central role in the timing of life-history transitions and can have organizational effects on the developing embryo, as embryonic exposure to corticosteroids results in widespread effects on growth and development that can permanently alter physiology and morphology (Denver, 1997; Denver and Crespi, 2006; Dennis et al., 2014). The altered activity in MCs of the OB, as well as the other phenotypic changes we observed in treated tadpoles, could be attributed to the elevation of corticosteroid levels in response to the predator stressor during embryonic development, to a self-remodelling of the neuronal circuitry, or to a combination of the two.

Plasticity in hatching time and the reduced baseline motility at 10 days may be regarded as a tadpole direct short-term response to the predation threat experienced as embryos (Ferrari and Chivers, 2010, 2013). By delaying their hatching time, treated embryos may have matured their brain and brain performances earlier than control siblings, thus showing a consistently higher neuronal sensitivity and responsiveness to predator cues in a later life stage. This implies also a long-term neuronal memory of embryonic experiences. Although experienced individuals may improve survival chances (Mathis and Smith, 1993), growing a more efficient anti-predator response in early life, by, for example, increasing the neurotransmission between ORNs and MCs, may incur energetic costs, because deviations from the developmental norm impose trade-offs in resource allocation (Steiner and Van Buskirk, 2008; Callahan et al., 2008; Auld et al., 2010; Dalesman et al., 2015). Actually, we observed a significantly smaller body size in treated hatchlings than in their control siblings, though development stage at hatching was almost identical for both. This may suggest that embryos exposed to the predator kairomone responded to the 'ghost of predation future' (Mathis et al., 2008; Ferrari and Chivers, 2010) by allocating their energy reserves to memorizing environmental information and improving their sensory system rather than to developing and growing up. Thus, neuronal plasticity may have entailed non-trivial costs during embryonic development, mainly because the neural tissue is one of the most metabolically expensive tissues (Laughlin et al., 1998; Attwell and Laughlin, 2001). Moreover, the inhibitory effect of embryonic kairomone exposure on growth and development was maintained and even exacerbated during ontogeny, at least until the age of 40 days, even in the absence of further exposure to predator cues. This could result in a delayed metamorphosis and/or in a smaller adult size for treated tadpoles compared with their control siblings, entailing possible fitness costs

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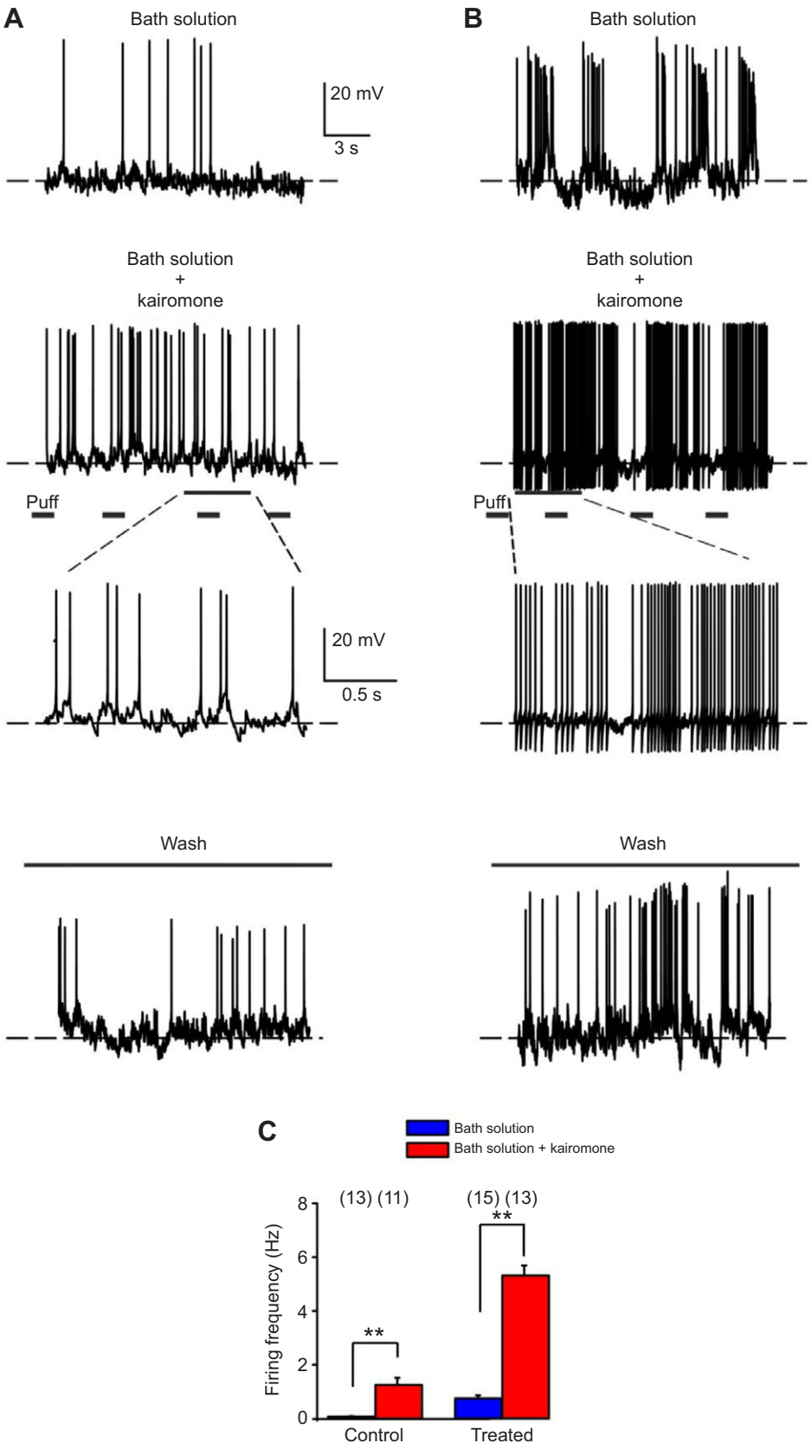

Fig. 4. Kairomone application induces a burst firing pattern in MCs of treated tadpoles only. (A) A representative trace of action potential firing recorded in current-clamp mode from a MC of a control tadpole before (bath solution) and after kairomone application (bath solution with dragonfly kairomone). Note that in baseline conditions the cell rarely reached the threshold for spiking; when kairomone was puffed, the firing frequency significantly increased, but was composed mostly of single spikes (see expanded time scale below the trace). In most cells, baseline activity recovered after kairomone solution washout. (B) Same as in A, but for the treated group. Note that both before and after kairomone solution application, the firing frequency was significantly higher than in the control group; in the representative cell shown, activity recovered after kairomone washout. (C) Mean firing frequency of recorded MCs from both control and treated tadpoles in the two experimental settings (bath solution and bath solution with dragonfly kairomone). *N*-values are given in parentheses; error bars represent s.e.m. with asterisks showing statistically significant differences (see Table 3 for statistics).

(Altwegg and Reyer, 2003). However, all clutches showed a similar response to embryonic kairomone exposure (delayed hatching time and reduced body size at hatching, as well as a higher neuronal activity at tadpole stage), implying little genotype by environment interactions in defensive phenotypic responses and suggesting a general adaptiveness of the ontogenetic plasticity in the population.

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In conclusion, our findings, by revealing extensive neuronal plasticity induced in tadpoles by predator chemical cues experienced during the embryonic stage, provide a novel insight into predator-induced patterns of ontogenetic phenotypic plasticity in anuran larvae.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
P.G., A.G. and F.B. conceived, designed and performed the experiments; P.G., D.R. and F.B. analysed the data; P.G., F.B. and A.G. wrote the paper; P.G. and D.R. reviewed and revised the paper; P.R. contributed reagents/materials/analysis tools, and critically reviewed and revised the paper. All authors gave their approval for submission.

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