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# Exposure to Pyriproxyfen pesticide, a juvenile hormone agonist, does not alter maternal care and reproduction in the European earwig

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## Abstract

Sublethal exposure to pesticides can alter the survival and reproduction of a wide range of non-target organisms. However, it remains unclear whether this exposure can alter behaviours that are often essential for long-term population dynamics and maintenance, such as parental care. In this study, we tested the effect of pyriproxyfen exposure (an insect growth regulator) on maternal care in the European earwig, an insect that is both used in pest control in pip-fruit orchards and considered a pest in stone fruit orchards. We exposed 424 females at doses either 10 times lower, equivalent or 10 times higher than normal application rates in French orchards. As maternal care can change over the weeks of family life, we exposed the earwig mothers at five different days before and after egg hatching. We then measured the expression of ten forms of maternal care towards eggs and juveniles, six non-caring behaviours, eggs and juveniles development, metabolic reserves in mothers at egg hatching and females' production of a terminal clutch. First, our results revealed that the three tested doses of pyriproxyfen were non-lethal and confirmed that maternal care decreased throughout both pre- and post-hatching family life. However, we did not detect any effect of pyriproxyfen on maternal care and non-care behaviours, eggs and juveniles development, quantities of lipids, proteins and glycogen in mothers at egg hatching, and on the production of a future clutch. Overall, these findings suggest that the maximal doses of pyriproxyfen authorized in French orchards is likely to have limited effects on the short- and long-term maintenance of populations of the European earwig, and raises fundamental questions about the nature of the link between juvenile hormone and parental care in insects.

**Keywords:** Juvenile Hormone; Egg care; Endocrine disruptor; Dermaptera; Pesticide

## Introduction

Pesticides are widely used in agriculture to control pest populations and increase yields production (Aktar et al. 2009; Guedes et al. 2016). However, the large-scale spraying of these chemicals in and around fields not only has an impact on pest survival (Torres and Bueno 2018) but also unintended lethal and sublethal effects on non-target species (Desneux et al. 2007; Royauté et al. 2015). These sublethal effects, which are broadly defined as physiological or behavioural effects on individuals that survive pesticide exposure (Desneux et al. 2007; Müller 2018), may affect a taxonomically large number of organisms (Desneux et al. 2007; Royauté et al. 2015) in which they can modify traits linked to reproductive outcome (number of offspring, developmental rate, hatching/emergence rate), behaviours (locomotion, mating, foraging, sociality), immunity and relationships with symbionts (Desneux et al. 2007; Müller 2018). These sublethal effects can have profound impacts on the fitness of each individual, population dynamics and ecosystem functioning (Desneux et al. 2007; Guedes et al. 2016) so it is timely and crucial to improve our current understanding of the diversity of these effects and the timing of their action, particularly for widely used pesticides.

Pyriproxyfen is a pesticide used worldwide in agriculture and horticulture, and in public health against fleas, mosquitoes and cockroaches (Sullivan and Goh 2008). This molecule is an agonist of juvenile hormone (JH), which is a major insect hormone that regulates metamorphosis, and typically plays a key role in the gonadotropic cycle (Sullivan and Goh 2008). It was long thought that its mode of action and specificity for the hormonal system of insects resulted in limited toxicity to mammals (but see Liu et al. 2020, Shahid and Saher 2020) while having major disruptive effects on insect development and reproduction, particularly when exposure occurs during early developmental stages (see review in Parthasarathy and Palli, 2021) and/or in individuals with low JH titers. For instance, several studies in psyllid, aphid and lepidopteran showed that the first developmental stages of larvae or eggs are more sensitive to pyriproxyfen, because either their exposure leads to an increased death rate or the dose required to reach a certain inhibition threshold is lower (Oouchi 2005; Richardson and Lagos 2007; Boina et al. 2010). In most lepidopteran species, exposure to pyriproxyfen during young larval stages also blocks subsequent moulting events, while exposure during late stages allows pupation but kills pupa before adult emergence (Parthasarathy and Palli 2021).

While many studies have investigated the sublethal effects of pyriproxyfen exposure in the juvenile stage in a wide variety of insects (and on many traits), less is known about the effects of exposure in the adult stage. This is surprising because adults can be exposed to JH agonists (including pyriproxyfen) in the context of pest control (Jindra and Bittova 2020) and the sensitivity and efficacy of JH analogues vary considerably between insects and life stages (Parthasarathy and Palli 2021). This makes it difficult to predict whether and how JH analogues shape specific traits in a given species and stage. Moreover, JH is a major hormone that regulates the expression of numerous fitness-related traits over an adult's lifetime. For instance, JH typically regulates vitellogenesis in the fat body and vitellogenin uptake by the ovaries (Wang and Davey 1993; Hartfelder 2000), a broad diversity of reproductive behaviours ranging from stimulating sexual pheromone production to regulating female receptivity (Ringo 2002), the expression of aggressive and dominant behaviours (Mathiron et al. 2019; Tibbetts et al. 2020), the activation of the immune response (Flatt et al. 2008; Contreras-Garduño et al. 2009) and the process of ageing (Hodkova 2008; Yamamoto et al. 2013). Moreover, the few studies that have investigated the sublethal effects of pyriproxyfen exposure at the adult stage report results suggesting that it can also disrupt embryogenesis and/or has ovicidal properties (Ishaaya and Horowitz 1995; Oouchi 2005; Boina et al. 2010). Hence, shedding light on the presence, nature, and intensity of sublethal effects of pyriproxyfen exposure during the adult stage may be of major importance in better understanding populations' dynamics, maintenance or elimination of pests and non-target species.

In addition to exploring the sublethal effects of pesticide exposure on physiology, a growing body of research points out that it is also necessary to investigate these effects on often neglected, yet essential, behaviours such as parental care (Royauté et al. 2015; Guedes et al. 2016; Meunier et al. 2020). This is because parental care is a common phenomenon in animals (including insects; Machado and Trumbo 2018, Meunier et al. 2022), where it is often critical to ensure eggs and/or juveniles development

(Klug and Bonsall 2014) and for which even subtle alterations can have major adverse transgenerational effects (Fleming et al. 2002; Champagne and Curley 2009; Thesing et al. 2015). An alteration of parental care due to pesticide exposure could thus be highly detrimental to offspring, which in turn could impair population dynamics, the maintenance of the exposed organisms and the long-term efficiency of this pesticide use (Cummings et al. 2010; Fong-McMaster et al. 2020). To date, however, the sublethal effects of pesticides on parental care were mostly investigated in a few studies conducted in rats and mice (Punzo 2003; de Castro et al. 2007; Stürtz et al. 2008; Venerosi et al. 2009; Udo et al. 2014; Keller et al. 2019) and a single insect species, the European earwig *Forficula auricularia* L. (Meunier et al. 2020; Mauduit et al. 2021). In particular, these two latter studies showed that maternal exposure to sublethal doses of deltamethrin - a pyrethroid pesticide - impairs maternal egg care (Meunier et al. 2020) and had only limited effects on post-hatching maternal care (Mauduit et al. 2021). Whereas none of these studies investigated the sublethal effects of pyriproxyfen exposure on parental care, a few studies demonstrated that pyriproxyfen exposure at the larval stage can alter other important behaviours in aquatic vertebrates and honeybees. For instance, pyriproxyfen exposure impaired inhibitory avoidance memory in Zebrafishes (Gusso et al. 2020) and swimming performances and prey capture abilities in mosquitoes (Caixeta et al. 2016), while the exposure of honeybee larvae produces adults with behavioural alterations, such as a lower expression of brood care and a higher rejection by nestmates (Fourrier et al. 2015).

In this study, we investigated the presence, nature, and intensity of sublethal effects of pyriproxyfen exposure in adult females of the European earwig *F. auricularia*. In this Dermapteran insect, females live for 18 months during which they produce up to two clutches without remating (Meunier et al. 2012; Tourneur and Meunier 2020). Mothers provide obligatory forms of care to their eggs for about 40 days during the winter and facultative forms of care to their juveniles for about 14 days in spring (Lamb 1976; Meunier et al. 2012; Thesing et al. 2015). This species is widespread in crop systems (Orpet et al. 2019a), where it is either used in pest control – such as in pip-fruits orchards - because its omnivorous feeding regime makes it an efficient predator against aphids, moths and psyllids (Moerkens et al. 2011; Dib et al. 2011), or as a pest – such as in stone fruits orchards – because it may also consume stone fruits and induce significant crop damages (Orpet et al. 2019a, b). This dual effect in orchards has fostered numerous studies exploring the lethal and physiological effects of several pesticides commonly applied in crops and vineyards in this species (Malagnoux et al. 2015b). These studies have not only documented the lethal effects of these pesticides on adult earwigs (French-Constant and Vickerman 1985; Colvin and Cranshaw 2010; Malagnoux et al. 2014) but have also reported some other behavioural and physiological effects of pesticide exposure. For instance, adult earwigs decreased predation activities after exposure to commonly used pesticides (e.g. acetamiprid, chlorpyrifos-ethyl, deltamethrin, and Spinosad; Malagnoux et al. 2015) and mothers showed a higher investment in future reproduction after exposure to deltamethrin (Mauduit et al. 2021). Whereas the effects of pyriproxyfen on the European earwig remain unknown, the behavioural and gonadotropic roles of JH have been reported in other Dermapteran species. In the ring-legged earwig *Euborellia annulipes*, for instance, the rate of circulating JH reaches a peak during oviposition, after which it rapidly decreases to allow the expression of maternal egg care (Rankin et al. 1995a). Moreover, the topical application of JH on females successfully restored oocytes production after the removal of the corpora allata (the gland that produces JH) in *Labiduria riparia* (Baehr et al. 1982), while it shortened the duration of maternal egg care and advanced the onset of the second gonadotrophic cycle in *E. annulipes* (Rankin et al. 1995b). Therefore, if the effects of JH on gonadotrophic/maternal care are comparable across Dermapteran species, we predict that exposure of *F. auricularia* adult females to pyriproxyfen alters both the expression of maternal care and their investment in future reproduction.

To test these predictions, we conducted a laboratory experiment in which we exposed *F. auricularia* females to pyriproxyfen doses either 10 times lower, equivalent or 10 times higher than normal application rates in French orchards (or to a control solution) and this exposure was done once for each female either on day 3, 20 or 36 after oviposition (i.e. during the egg care period) or on day 1 or 5 after egg hatch (i.e. during the post-hatch family life period). We subsequently maintained animals under standard conditions and measured the effects of exposure on ten classical forms of pre- and post-hatching maternal care, six non-social behaviours, and the level of metabolic reserves (lipids, proteins and

glycogen) remaining in mothers at egg hatching. We subsequently investigated the effects of exposure on short-term reproduction in terms of egg hatching rate, juveniles' mean weight at hatching and juveniles' survival during family life, as well as on long-term reproduction in terms of a second (and terminal) clutch production, delay until this second clutch production and the number of second clutch eggs produced.

## Materials and methods

### Animal sampling and breeding

The experiment involved a total of 424 *F. auricularia* females (clade B; Wirth et al. 1998, González-Miguéens et al. 2020) that were field-sampled using wood traps in apple and pear orchards in Pont-de-Ruan, France (Lat 47.245373, Long 0.578932), in July 2020. On the day of field sampling, we set up males and females in plastic terrariums (balanced sex-ratio) lined with moistened sand and containing egg cardboard to serve as a shelter for individuals. Each terrarium received an *ad libitum* amount of homemade food (consisting mainly of a mixture of pollen, cat food and bird seeds; details in Kramer et al. 2015) that was changed once a week, and was maintained under a 12:12 light:dark cycle at 18:20°C, respectively. This setup allowed for uncontrolled mating in the experimental population (Sandrin et al. 2015). Five months later (in November 2020), we isolated females to mimic their natural reproductive behaviour and stimulate oviposition (Kölliker 2007; Körner et al. 2018). We transferred each female in a Petri dish (diameter 5 cm) lined with moistened sand and maintained under constant darkness at 10°C. We then checked each female daily to record the date of oviposition. On that day, we removed food from the Petri dish as mothers typically stop their foraging activity between egg-laying and egg hatching (Kölliker 2007) and started the experimental process described below.

### Overview of the experiment

The experiment started on the day of oviposition. At that time, we randomly distributed each female to one of five treatments reflecting either an exposure during the period of egg care (treatments 1 to 3) or during the period of post-hatching family life (treatments 4 and 5). In particular, we exposed females either at (1) day 3 after oviposition (n= 106 females), (2) day 20 after oviposition (n= 107 females), (3) day 36 after oviposition (n= 104 females), (4) day 1 after egg hatching (n= 54 females) or at (5) day 5 after egg hatching (n= 53 females).

Three days after oviposition, we counted the number of eggs produced by the 317 females involved in the treatments reflecting exposure during the period of eggs care (i.e. treatments 1, 2 and 3). We then immediately trimmed their clutch size to 25 (mean  $\pm$  SE number of eggs produced =  $39 \pm 8.7$ ) to limit uncontrolled variation in maternal egg care due to uncontrolled variation in clutch size. In the few cases where females produced between 20 and 25 eggs (n = 18; 5.6% of the 317 clutches), we added extra-numerary eggs from another clutch to reach 25. This was possible because earwig mothers do not discriminate against foreign eggs (Van Meyel et al. 2019). We then exposed these 317 females to a pyriproxyfen solution either 3, 20 or 36 days after oviposition (see exposure details below) and subsequently measured maternal egg care and non-care behaviours. We then checked each female daily to record the date of egg hatching. On that day, we immediately transferred the Petri dishes containing each mother and its newly hatched juveniles (called nymphs) to a climate cabinet under 12: 12 light: dark cycle and 18:20°C, with *ad libitum* amount of homemade food that was changed twice a week. This standard process allows juveniles development during earwig family life (Meunier et al. 2012). One day after egg hatching, we counted the number of newly hatched nymphs, weighed a group of ten random nymphs per brood (or all nymphs if less than 10 nymphs were available) and then removed these post-hatching families from the rest of the experiment.

Next to the 317 females detailed above, the 107 females involved in the treatments reflecting exposure during post-hatching family life (i.e. treatments 4 and 5) were maintained with their original number of eggs under the standard laboratory conditions detailed above until one day after egg hatching. On that day, we counted the number of newly hatched nymphs, trimmed each brood to 15 nymphs (mean  $\pm$  SE number of nymphs produced =  $23.6 \pm 8.5$ ) and finally transferred the mother and its 15 nymphs to a

new large Petri dish (diameter 14 cm). Because maternal acceptance of foreign nymphs is unclear in *F. auricularia*, we did not mix nymphs from different families and kept 18 (16%) of the 107 females with their initial number of 10 to 14 nymphs. We then exposed these 107 females to one of the pyriproxyfen solutions (see details below) either 1 or 5 days after egg hatching and subsequently measured post-hatching maternal care and non-care behaviours, as well as females future reproduction (see details below). We then isolated each mother 14 days after egg hatching and maintained them under the same temperature and under complete darkness to allow the production of a second (and terminal) clutch (Meunier et al. 2012). The weighing was conducted to the nearest 0.01 mg using a microbalance (OHAUS® Discovery DV215CD).

### **Pyriproxyfen exposure**

We exposed each of the 424 females to one of four pyriproxyfen solutions using a standard protocol mimicking natural earwig exposure to pesticides in Orchards (Malagnoux et al. 2015a; Meunier et al. 2020; Mauduit et al. 2021). We prepared the four pyriproxyfen solutions by diluting the powdered form of pyriproxyfen (Sigma-Aldrich, Ref. 101819775) in absolute ethanol (Fisher Chemicals, E.0665EDF.17) to obtain concentrations of either 75 µg/mL (hereafter called “High”), 7.5 µg/mL (“Normal”), 0.75 µg/mL (“Low”) or 0 µg/mL (“Ethanol”). The three first concentrations were chosen because they are 10 times higher, equivalent or 10 times smaller than the normal application rate (NAR) authorized in French orchards (0.3g/ha), respectively (Malagnoux et al. 2015b). To carry out each exposure, we uniformly deposited (with rotation movement) 88 µL of the corresponding pyriproxyfen solution on the bottom of a Petri dish (diameter 3.5 cm, changed for each individual), allowed it to dry for 30 minutes under a hood, and then deposited each female in the Petri dish newly lined with pesticide (or solvent) residuals. We kept these females in these Petri dishes for four hours at room temperature and under bright light to increase the walking activity of this luciferous species (Meunier et al. 2020; Mauduit et al. 2021). This process mimics natural earwig exposure to pesticides because pesticide spraying typically occurs during daytime in orchards and earwigs are nocturnal, so individuals may only encounter pesticides by walking on their residues on leaf and soil surfaces (Fountain and Harris 2015; Orpet et al. 2019a). Furthermore, previous studies demonstrated that this experimental process of exposure triggers altered behaviours when *F. auricularia* females are exposed once to sublethal doses of other pesticides, such as deltamethrin, spinosad, acetamiprid and chlorpyrifos (Malagnoux et al. 2015a; Meunier et al. 2020; Mauduit et al. 2021). Overall, this process led us to expose 424 females, of which 317 were exposed before oviposition (n=78, n=80, n=78 and n=82, exposed to the Ethanol, Low, Normal and High treatments, respectively) and 107 after egg hatching (n=27, n=26, n=25 and n= 29, exposed to Ethanol, Low, Normal and High treatments, respectively). Each of these females was exposed to pyriproxyfen or ethanol residuals only once in their life.

### **Egg care measurements**

We measured five classical forms of egg care in the 317 females exposed before egg hatching. We used protocols already established in earwigs (Thesing et al. 2015; Van Meyel et al. 2019; Meunier et al. 2020) to record the [1] occurrence of egg gathering both 2h and 5 days after exposure, [2] frequency of contacts between mothers and eggs 2 days after exposure, [3] level of egg defence after a simulated predator attack 2 days after exposure, [4] delay of egg retrieval after this simulated predator attack and the [5] mean distance between mothers and eggs 5 days after exposure. [1] The occurrence of egg gathering reveals the likelihood of mothers actively collecting their experimentally dispersed eggs. While the mothers were isolated for exposure, we levelled the sand in their original Petri dishes and evenly distributed their 25 eggs along the edge of a 2 cm diameter circle. The eggs were at least one egg length apart from each other. At the end of the 4-hour exposure, we returned each female to the centre of the circle and recorded whether all eggs were gathered in a circle of 1 cm diameter 2 hours later. The same process was repeated on day 5 after exposure, i.e. we isolated each female, distributed their eggs along the edge of a 2 cm diameter circle, returned each female to the centre of this circle immediately afterwards, and recorded the occurrence of egg gathering after 2 hours. [2] The frequency of contact between mothers and eggs reflects the number of observations during which a mother was in contact with its eggs over 30 minutes. We obtained this number using a scan-sampling process during which we

recorded every minute whether females were touching/cleaning eggs with their mouthparts (i.e. grooming) and/or with antennae (i.e. antennation). We conducted this test on day 2 after exposure and via direct observations through the lids of the Petri dishes. [3] The level of egg defence shows females' willingness to protect their eggs from predator attacks. Just after measuring the frequency of the contact between mother and eggs, we gently opened each Petri dish, standardly poked each female on the pronotum with a glass capillary and recorded the number of pokes required until they moved more than 1 body length away from the clutch. High values of egg defence (poke number) show high maternal investment in egg care and vice versa. [4] The delay of egg retrieval shows the delay after which a female returns to its eggs after being chased away by a simulated predator attack. Immediately after the measurement of egg defence, we recorded the number of seconds it took for each female to touch one of her eggs again. We stopped recordings after 480 s. Long delays of egg retrieval indicate low maternal egg care and vice versa. Finally, [5] we measured the distance between the mother head and the centre of its largest pile of eggs on day 5 after exposure via direct measurements through the lids of the Petri dishes.

### **Nymph care measurements**

We then measured four classical forms of post-hatching maternal care in the 107 females exposed after egg hatching. We used a series of standard protocols (Meunier et al. 2012; Thesing et al. 2015; Mauduit et al. 2021) to measure the [1] likelihood of family reunification, [2] frequency of mother-offspring contacts, [3] level of nymph defence against a simulated predator attack and the [4] level of maternal food provisioning. [1] The likelihood of family reunification shows the likelihood that mothers would return to their clutch of nymphs after experimental isolation. While the mothers were exposed to pyriproxyfen (or ethanol) residues and the pupae were weighed, we dug two small holes in the sand of their original Petri dish (distance between holes = 7 cm) and partially covered them with a plastic cap to form two artificial nests. We then placed all nymphs in one of the two nests (we alternated the chosen nest between the tests) and the associated mother in the centre of the Petri dish. One hour later, we determined whether the mother was with the largest group of nymphs (either in one of the artificial nests or outside) and if so, we gave her a score of 1 (0 otherwise). [2] Just like for pre-hatching care, the frequency of mother-offspring contacts was measured 2 days after exposure and reflects the number of observations during which a mother was in contact with a juvenile over 30 minutes. We obtained this number using a scan-sampling process during which we recorded every minute whether females were touching/cleaning the body of their nymphs with their mouthparts (i.e. grooming), had mouth-to-mouth contacts with nymphs (i.e. stomodeal trophallaxis) and/or were touching nymphs with their antennae (i.e. antennating). We conducted this test via direct observations through the lids of original Petri dishes. [3] The level of nymph defence reflects females' willingness to protect their nymphs from predator attacks. Just after measuring the frequency of the contact between mother and nymphs, we gently opened each Petri dish and standardly poked each female on the pronotum with a glass capillary and recorded the number of pokes required until they moved more than 1 body length away from the group of nymphs. [4] The level of maternal food provisioning was measured using a 4-steps process (Kramer et al. 2015): we first removed food from the Petri dish 2 days after exposure, we then isolated mothers in new Petri dishes (diameter 7 cm) and provided them with one green-coloured pollen pellet for four hours on day 4 after exposure (Staerkle and Kölliker 2008), after which we reassembled the fed mothers with their nymphs in their original Petri dish for 24h to allow family interactions. We finally counted the number of green-nymphs, i.e. nymphs that had consumed the green-coloured food provided by the mother. This measurement relies on the fact that first instar nymphs are translucent and it is thus possible to record their consumption of coloured food through the cuticle (Staerkle and Kölliker 2008).

### **Non-care behavioural measurements**

We measured four behaviours that were not associated with care, either in the 317 females exposed before egg hatch or in the 107 females exposed after egg hatch, or in both. These measurements relied on standard protocols (Meunier et al. 2020; Mauduit et al. 2021) and reflected females' (1) general activity in absence of eggs or juveniles, (2) food consumption, (3) self-grooming and (4) resting. (1) We measured the general activity of females in absence of eggs (or juveniles) on day 2 after exposure. Just

after all the other measurements conducted on that day (see above), we gently transferred each female to an empty circular arena (diameter 18 cm) held between two glass plates on an infrared light table. We then video recorded females for 30 minutes under darkness – but with infrared light (BASLER BCA 1300, Germany; Media Recorder v4.0, Noldus Information Systems, Netherland) and defined its level of activity as the total distance walked by the female during this time. This distance was automatically extracted from our videos using the software ToxTrac v2.92 (Rodriguez et al. 2018). (2) We measured the level of females' food consumption at the beginning of the measurement of food provisioning in the 107 females exposed after egg hatching. On day 4 after exposure, we weighed each of the pollen pellets provided to the isolated mothers before and after the 4h-contact with the mothers to the nearest 0.01 mg and defined food consumption as the difference between the two measurements. Because subtle variation in humidity and temperature may induce uncontrolled variation in pellets weight (Mauduit et al. 2021), we added a control treatment to shed light on this variation by handling the pellet the same way but without an earwig mother in the Petri dish. (3) Self-grooming is a major behaviour in insects (including earwigs) which allows individuals to both remove dirt and pathogens, and apply self-secreted chemicals on the cuticle to enhance protection against desiccation and mediate communication with conspecifics (Blomquist and Bagnères 2010; Weiß et al. 2014; Boos et al. 2014). We measured the frequency of self-grooming on day 2 after exposure in females exposed before and after egg hatching, while we were recording the frequencies of mother-egg and mother-nymph interactions (see above). During the 30 minutes of scan sampling, we recorded every minute whether a female was touching or scratching one of its body parts (i.e., antennae, legs, abdomen, cerci) with its mouthparts and/or legs. (4) The frequency of resting in presence of eggs or nymphs reflected the total number of times we observed a female remained motionless in presence of its descendants. It was measured in the same females, at the same time and following the same scan sampling process as for self-grooming.

### **Offspring development and females' reproduction and survival**

In addition to the behaviours detailed above, our experimental design allowed us to investigate the effects of pyriproxyfen exposure on a series of other traits related to egg development (hatching rate and nymph weight at hatching), offspring survival during family life, as well as females' investment in future reproduction. (1) We measured the hatching rate in females exposed before egg hatching only (i.e. treatments 1, 2 and 3), by counting the number of nymphs present in the Petri dish on the day following the first egg hatching (earwig eggs typically hatch within one day; Koch and Meunier 2014). We defined the hatching rate as the number of newly hatched nymphs divided by the number of eggs at the time the experiment was set up. (2) We measured the mean nymph weight at hatching in females exposed before egg hatching only, by weighing a random group of ten nymphs (or all the nymphs if less than ten nymphs were available) per clutch on the day following egg hatching. We then divided this group weight by the number of weighted nymphs to obtain the mean nymph weight per clutch. (3) To measure nymph survival until the end of family life, we counted the number of nymphs alive 14 days after egg hatching in families where females were exposed before and after hatching. Finally, (4) we measured maternal investment in second clutch production in females exposed after egg hatching only (i.e. treatments 4 and 5). We monitored each female isolated 14 days after egg hatching on each subsequent day to record (a) whether females indeed produced a second clutch, (b) the date of this second clutch production and (c) the number of eggs produced in this second clutch. The delay until the second clutch production was calculated as the difference between the date of the first clutch egg hatching and the date of the second clutch production.

### **Metabolic quantification in mothers at egg hatching**

We measured the levels of proteins, lipids and glycogens in 124 females at egg hatching. These females were evenly distributed between treatments ( $n = 33$ ,  $n = 29$ ,  $n = 32$ ,  $n = 30$  exposed to Ethanol, Low, Normal and High, respectively) and between days of exposure ( $n = 41$ ,  $n = 43$ ,  $n = 40$  exposed on day 3, day 20 and day 36 after oviposition, respectively) to obtain 9 to 12 replicates per combination. We sampled these females on the day following egg hatching, then weighted them, transferred them individually in 2 mL Eppendorf tubes, plunged them in liquid nitrogen and finally stored them at  $-80^{\circ}\text{C}$  until metabolic quantifications.



We performed metabolic quantifications following standard protocols previously developed in the European earwig (Suchail et al. 2018; Le Navenant et al. 2021). First, we homogenized on ice each whole earwig body in 10% low-salt buffer containing 10mM Tri-Hcl (pH 7.3) and 10mM NaCl and then centrifuged for 10 min at 3000 g (Suchail et al. 2018). We used the resulting supernatant to measure protein, lipid and glycogen contents. We measured protein content using Bradford protein assay (BIORAD #50000204) from 1 $\mu$ L of supernatant and compared it to a standard curve of Bovine gamma-globulin (0.125-2 mg/ml). We measured total lipids by adding 6 $\mu$ L of either supernatant or standard solution (oil) to concentrated sulfuric acid (294  $\mu$ L), homogenizing the solution, placing it in water at 98 °C for 10 min and finally cooling it on ice for 5 min. A 700  $\mu$ L of phospho-vanillin reagent was added to each extract or lipid standard. After homogenization and incubation at 37 °C for 15 min, we cooled samples on ice and the absorbance was recorded at 540 nm. Finally, we measured glycogen content using the method based on enzymatic hydrolysis of glycogen by amyloglucosidase (EC 3.2.1.3). We mixed 250  $\mu$ L of crude extract (v/v) to a solution of trichloroacetic 4% acid for deproteinization. We then centrifuged the solution at 3000 $\times$ g for 1 min at 4 °C. We added two volumes of 95% ethanol to precipitate the glycogen present in the supernatant, which was pelleted by centrifugation at 5000  $\times$ g for 5 min at 4°C. The pellet was then dried for 2 h at room temperature to remove ethanol. Once dried, we incubated the glycogen pellet for 2 h at 60 °C in 500  $\mu$ L of 0.2 M sodium acetate pH 5.2 containing 7 IU (International Units) of amyloglucosidase (Sigma #10115. We then cooled down the resulting solution on ice and measured the amount of glucose generated from glycogen using the Glucose GOD-PAP method (Abliance #87109) adapted to a 96-well microplate format. We left the resulting solution with 250  $\mu$ L of Glucose GOD-POD and 25  $\mu$ L of sample at room temperature for 20 min and then measured glucose via absorbance at 505 nm. We finally calculated the amount of glucose from a standard curve ( $A_{505} = f[\text{glucose}]$ ) containing pure glucose as a standard treated with the same conditions. Because the final values were included, the amount of glycogen was corrected for the glucose content in samples that were not incubated with amyloglucosidase.

### Statistical analysis

We performed all statistical analysis using the R software v4.1.1 (<https://www.r-project.org/>) loaded with *DHARMA* (Hartig 2020), *car* (Fox and Weisberg 2019), *emmeans* (Lenth 2021) and *survival* (Therneau 2020) packages. We analysed our 25 measurements using a series of 10 generalized linear models (GLM) fitted with either binomial or Poisson error distributions, 14 linear models (LM) and a Cox proportional hazards regression model (Table 1). In these 25 models, we used the concentration of pyriproxyfen exposure (0.00, 0.75, 7.50 or 75.00  $\mu$ g/mL), the day of exposure (either 3, 20 and 38 before egg hatching, or 1 and 5 after egg hatching) and the interaction between these two parameters as explanatory factors. The 10 GLMs included either egg gathering (Binomial error), frequency of mother-egg contacts (Poisson error corrected for overdispersion), egg defence (Poisson error), egg gathering (Binomial error), mother-egg distance (Poisson error corrected for overdispersion), self-grooming frequency before hatching (Poisson error corrected for overdispersion), family reunification (Binomial error), food provisioning (Binomial error corrected for overdispersion and with the ratio of coloured nymphs entered using the *cbind* function), nymphs survival rate until the end of family life (Binomial error corrected for overdispersion and with the rate entered using the *cbind* function) or the likelihood to produce a second clutch (Binomial error with a *cloglog* link function to correct for unbalanced representation of 0 and 1) as a response variable. Conversely, the response variable in the 14 LMs was either the resting frequency before hatching, the general mother activity before hatching (log+1-transformed), the quantities of proteins, lipids and glycogens in mothers at egg hatching, the number of nymphs at egg hatching (log+1-transformed), the mean nymph weight at egg hatching, the frequency of mother-nymphs contacts (log+1-transformed), the level of nymph defence (log+1-transformed), the resting frequency after egg hatching, the frequency of self-grooming after egg hatching, mother general activity after egg hatching (log+1-transformed), delay until 2<sup>nd</sup> clutch production or the number of eggs in the 2nd clutch as a response variable. Finally, the egg retrieval time was tested using a Cox proportional hazards regression model allowing for censored data, that is, mothers that did not retrieve their eggs at the end of the experiment. We checked that the assumptions of the 25 statistical models were met using the *DHARMA* package (Hartig 2020) and log+1-transformed the response variable when required (see above). When necessary, we conducted pairwise

comparisons using estimated marginal means of the models and with p-values corrected for multiple testing using Tukey methods (*emmeans* package).

## Results

Overall, 7 of the 424 (1.6%) tested females died naturally ( $n = 5$ ) or accidentally, ( $n = 2$ ) during the experiment, while 2 (0.5%) were lost and 1 (0.25%) accidentally lost its clutch. The 7 females that died were evenly distributed among the treatments (Solvent,  $n = 1$ ; Low,  $n = 1$ ; Normal,  $n = 5$  - whose 2 accidental deaths) and the 10 females were excluded from the statistical analysis detailed below.

### Pyriproxyfen exposure during the period of egg care

Maternal exposure to each dose of pyriproxyfen (whether at 3, 20 or 36 days after oviposition) affected none of the 6 measured forms of egg care (Table 1; Figure 1). In particular, pyriproxyfen exposure did not alter the likelihood that mothers would collect their experimentally dispersed eggs 2h and 5 days after exposure, the frequency of mother-egg contacts 2 days after exposure, the level of egg defence against a simulated predator attack 2 days after exposure, the speed at which females retrieved their eggs after this simulated predator attack and the distance between the mother and its clutch of eggs 5 days after exposure (Table 1). Similarly, pyriproxyfen exposure at 3, 20 or 36 days after oviposition altered none of the 3 non-care behaviours (i.e. resting, self-grooming and general activity in absence of eggs) measured 2 days after exposure (Table 1; Figure 2), none of the 3 energetic reserves (proteins, lipids and glycogen) measured in mothers at egg hatching (Tables 1 & 2), as well as shaped neither the number of nymphs produced nor the mean weight of these nymphs (Tables 1 & 2).

Independent of pyriproxyfen exposure, three of the females' behaviours changed during egg development (Table 1). The likelihood of mothers gathering experimentally dispersed eggs was overall higher when measured at day 3 compared to days 20 and 36 (Fig 1; Posthoc tests: d3 vs d20:  $p=0.0001$ ; d3 vs d36:  $p<0.0001$ ; d20 vs d36:  $p=0.891$ ). For clarity, here and in the rest of the text, the day of measurement refers to the day of pesticide exposure, as the two are closely related and only 0-2 days apart depending on the measurement (Table 1). The level of egg defence was overall lower when measured at day 36 compared to days 3 and 20 (Fig 1; Posthoc tests: d3 vs d20:  $p=0.715$ ; d3 vs d36:  $p=0.004$ ; d20 vs d36:  $p=0.036$ ), and the duration of egg retrieval was overall longer when measured at day 36 compared to day 20 (Fig 1; Posthoc tests: d3 vs d20:  $p=0.412$ ; d3 vs d36:  $p=0.077$ ; d20 vs d36:  $p=0.026$ ). Moreover, the resting frequency of mothers in presence of eggs was overall higher when measured at day 3 compared to days 36, with an intermediate duration when measured at day 20 (Fig 2; Posthoc tests: d3 vs d20:  $p=0.793$ ; d3 vs d36:  $p=0.023$ ; d20 vs d36:  $p=0.113$ ). The self-grooming frequency in presence of eggs was overall lower when measured at day 36 compared to days 3, with an intermediate duration when measured at day 20 (Fig 2; Posthoc tests: d3 vs d20:  $p=0.093$ ; d3 vs d36:  $p<0.001$ ; d20 vs d36:  $p=0.099$ ). Females' general activity in absence of eggs was also overall higher when measured at day 36 compared to days 3 and 20 (Fig 2; Posthoc tests: d3 vs d20:  $p=0.103$ ; d3 vs d36:  $p=0.065$ ; d20 vs d36:  $p<0.001$ ). Finally, the day of measurement shaped none of the 3 energetic reserves (proteins, lipids and glycogen) measured in mothers at egg hatching, nor influenced the number of nymphs produced nor the mean weight of these nymphs (Tables 1 & 2).

### Pyriproxyfen exposure during post-hatching family life

As for egg care, maternal exposure to each dose of pyriproxyfen (whether at 1 or 5 days after egg hatching) affected none of the 4 measured forms of post-hatching care (Table 1; Figure 3): it did not alter the likelihood that mothers would return to their clutch of nymphs 1h after exposure (family reunification), the frequency of mother-nymphs contacts 2 days after exposure, the level of nymph defence against a simulated predator attack 2 days after exposure, and the level of maternal food provisioning 4 days after exposure (Table 1). Similarly, such a pyriproxyfen exposure altered none of the 3 non-care behaviours (i.e. resting, self-grooming and general activity in absence of nymphs) measured 2 days after exposure (Table 1; Figure 4) and did not affect the number of surviving nymphs at the end of family life (Table 1 & 2).

Four females' behaviours changed during the development of their nymphs (Table 1). Both the

likelihood that mothers would return to their clutch of nymphs 1h after exposure and the frequency of contacts between a mother and its nymphs 2 days after exposure were higher when pyriproxyfen exposure occurred at day 1 compared to day 5 after egg hatching (Figure 3), whereas the level of maternal food provisioning was overall lower when pyriproxyfen exposure occurred at day 1 compared to day 5 (Figure 3). The day of exposure after egg hatching did not influence the mother's general activity nor the nymph's survival at the end of family life (Tables 1 & 2).

## Discussion

Whereas a growing number of studies emphasizes that sublethal exposure to pesticides can alter the survival and reproduction of a broad diversity of non-target organisms (Desneux et al. 2007; Guedes et al. 2016; Müller 2018), these effects on critical behaviours such as parental care remain less clear. In this study, we addressed this important issue by exposing earwig females at pyriproxyfen doses either 10 times lower, equivalent or 10 times higher than normal application rates in French orchards and then measured the expression of ten forms of maternal care towards eggs and juveniles, six non-caring behaviours, eggs and juveniles' development, metabolic reserves in mothers at egg hatching and females' production of a future terminal clutch. As maternal care can change over the weeks of family life, pyriproxyfen exposure took place on one of five different days before and after egg hatching. Overall, our results confirmed the presence of an age-dependent expression of maternal care in the European earwig, which decreases both between egg-laying and egg hatching, and between egg hatching and the end of family life. However, we did not detect any effect of pyriproxyfen exposure on the expression of pre- and post-hatching maternal care and non-care behaviours by females, in the level of metabolic reserves in mothers at egg hatching, and maternal investment in future reproduction.

Our study did not allow to detect an effect of pyriproxyfen exposure on the expression of multiple forms of maternal care towards eggs and juveniles. This reveals that maximal doses of pyriproxyfen authorized in French orchards are unlikely to alter critical behaviours such as maternal care in the European earwig, unlike other pesticides such as deltamethrin (Meunier et al. 2020). This was somewhat surprising, as previous works reported a direct link between juvenoids (of which pyriproxyfen is an agonist of the intracellular JH receptor Met) and maternal care in other earwigs species: maternal care is associated with very low concentrations of juveniles hormone in females of the ring-legged earwig *Euborellia annulipes* (Rankin et al. 1995b) and the shore earwig *Labidura riparia* (Vancassel et al. 1984), and topical applications of juveniles hormone on mothers after oviposition shorten the duration of maternal egg care in the ring-legged earwig *E. annulipes* (Rankin et al. 1997). We formulate three hypotheses to explain this apparent discrepancy. First, pyriproxyfen may not have the same action as juvenile hormone (JH) in the European earwig. This hypothesis is unlikely to explain our results, as pyriproxyfen is known to have analogous effects of JH in a taxonomically diverse number of insects (Hatakoshi 1992; Ishaaya and Horowitz 1995; Liu 2003; Richardson and Lagos 2007; Parthasarathy and Palli 2021). Moreover, the transcription-inducing activity of pyriproxyfen is equivalent to that of JHIII in a luciferase reporter gene assay for JHAs in HEK297T cells transiently expressing a *Drosophila* receptor Met and its heterodimerization partner Tai (Yokoi et al. 2020), and pyriproxyfen was tested for direct interaction with the *Tribolium* Met (Charles et al. 2011) or *Drosophila* Gce proteins (Jindra et al. 2015) and in both cases, pyriproxyfen was a better competitor than JHIII itself. Although these results suggest that pyriproxyfen has good affinity for the Met receptor, the exact nature of receptor binding may differ from that of natural JH. The second hypothesis is that JH may be involved in maternal care and oogenesis in *F. auricularia*, but the doses of pyriproxyfen allowed in agriculture (and used in our study) are not high enough to trigger phenotypic changes in these traits. This would suggest that residual application of pyriproxyfen at 75 µg/mL is not sufficient to trigger enough receptors and lead to behavioural or physiological alterations, whereas topical applications of 50 µg of JH is enough to shorten the duration of maternal care in *E. annulipes* (Rankin et al. 1997). Finally, the third hypothesis is that JH does not mediate maternal care in *F. auricularia*, at least not in the way we thought in the first instance. This is in line with a recent review shedding light on the contrasting links between JH and maternal care across insects (Trumbo 2018). For instance, maternal egg care is associated with low JH titers in *E. annulipes* and *L.*

*riparia* (Vancassel et al. 1984; Rankin et al. 1995b), whereas the application of a JH antagonist (precocene) leads to the termination of egg-brooding in the burrower bug *Sehirus cinctus* (Kight 1998), and post-hatching parental care is associated with high JH titers in the burying beetles *Nicrophorus spp* (Trumbo and Rauter 2014). If this latter physiological pattern applies to *F. auricularia*, exposure to pyriproxyfen would not be able to activate the JH receptors already saturated by naturally high levels of circulating JH and its exposure would thus be unable to affect maternal care. This last hypothesis, however, is at odds with findings from a recent transcriptomic study, in which JH signalling was higher in females before egg hatching compared to after post-hatching family life (Wu et al. 2020).

Our results also did not shed light on the effect of pyriproxyfen exposure on females' future reproduction. Specifically, pyriproxyfen exposure after egg hatching did not influence the likelihood to produce a second clutch, the number of days between the first and the second oviposition and the size of the second clutch. As for maternal care, this finding emphasizes that maximal doses of pyriproxyfen authorized in French orchards are unlikely to alter the reproduction of *F. auricularia* females. Nevertheless, this result was surprising for two main reasons. First, because reproduction is one of the most sensitive traits to pesticide exposure in arthropods (Desneux et al. 2007) and a previous study in *F. auricularia* showed that maternal exposure to deltamethrin (a pyrethroid insecticide) after egg hatching augmented their likelihood to produce a second clutch, reduced the delay of 2nd clutch production and increased the number of 2nd clutch eggs (Mauduit et al. 2021). This was also surprising because JH typically stimulates vitellogenesis (Vg) and ovarian maturation in most insects species (Hartfelder 2000)(including earwigs, see Vancassel et al. 1984, Rankin et al.1995a, 1997) and a previous study demonstrated that the application of JH advances the onset of the second gonadotrophic cycle in *E. annulipes* (Rankin et al. 1997). Our finding is unlikely to reflect that JH and Vg are decoupled in the European earwig, as a recent study demonstrated antagonistic JH-Vg regulation in post-hatching parental care in this species (Wu et al. 2020), and instead calls for future studies exploring the link between JH, Vg and other hormones on ovarian maturation and reproduction in *F. auricularia* females. It also emphasizes that the sole alteration of JH titers via residual exposure to the three tested concentrations of JH-agonists (pyriproxyfen) is unable to alter the regulation of females' future reproduction in *F. auricularia*.

Overall, our results provide no evidence for sublethal effects of pyriproxyfen on the expression of pre- and post-hatching maternal care, egg and juveniles development and maternal investment in future reproduction in the European earwig. Importantly, these results were independent of the tested doses of pyriproxyfen and the timing of maternal exposure during the periods of egg and juvenile care. These data thus indicate that the maximal doses of pyriproxyfen authorized in French orchards is likely to have limited direct effects on the maintenance of populations of the European earwig (when applied on adults), and may thus not hamper their use in pest-control in pip-fruits orchards (Moerkens et al. 2011; Dib et al. 2011), or trigger their elimination in stone fruit orchards where they are typically considered pests (Orpet et al. 2019a, b). This is consistent with the results of a recent study in the collared earwig *Euborellia annulipes* which showed that exposure of adults to pyriproxyfen had only limited effects on food consumption and no impact on their survival (Potin et al. 2022). More generally, these results show that the sublethal effects of pesticides exposure on parental care, which have been reported mostly in rats and mice (Punzo 2003; de Castro et al. 2007; Stürtz et al. 2008; Venerosi et al. 2009; Udo et al. 2014; Keller et al. 2019), are not necessarily present in arthropods and/or may tightly depend on the type of pesticides (see eg. Meunier et al. 2020, Mauduit et al. 2021). This emphasizes that publishing studies reporting non-significant effects of pesticide exposure (while mimicking natural processes of exposure) is essential to improve our general knowledge on pesticides use and their potential risks in integrated pest management programs. It also urges us to study these sublethal effects in a wider range of taxonomically diverse species (Royauté et al. 2015; Guedes et al. 2016; Meunier et al. 2020) to shed light on the general impact of these effects on the dynamics of species that could be of key agricultural, economic, and ecological importance. In addition to the ecotoxicological impacts of our results about pyriproxyfen exposure in the European earwig, the fact that this JH agonist does not alter maternal care raises fundamental questions about the endocrine regulation of this behaviour and more specifically, the nature of the link between JH and parental care across Dermaptera and arthropods (Trumbo 2018, 2019). Overall, this study thus emphasizes the twin benefits of studying exposure to insect growth regulators in insects: assessing the effects of pesticides on

non-target species and improving our general understanding of the hormonal regulation of critical behaviours and reproductive parameters.

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**Availability of data and materials.** The 3 datasets analyzed in the current study and the associated R script are available as supplementary files following this link: <https://doi.org/10.1007/s11356-022-20970-z>

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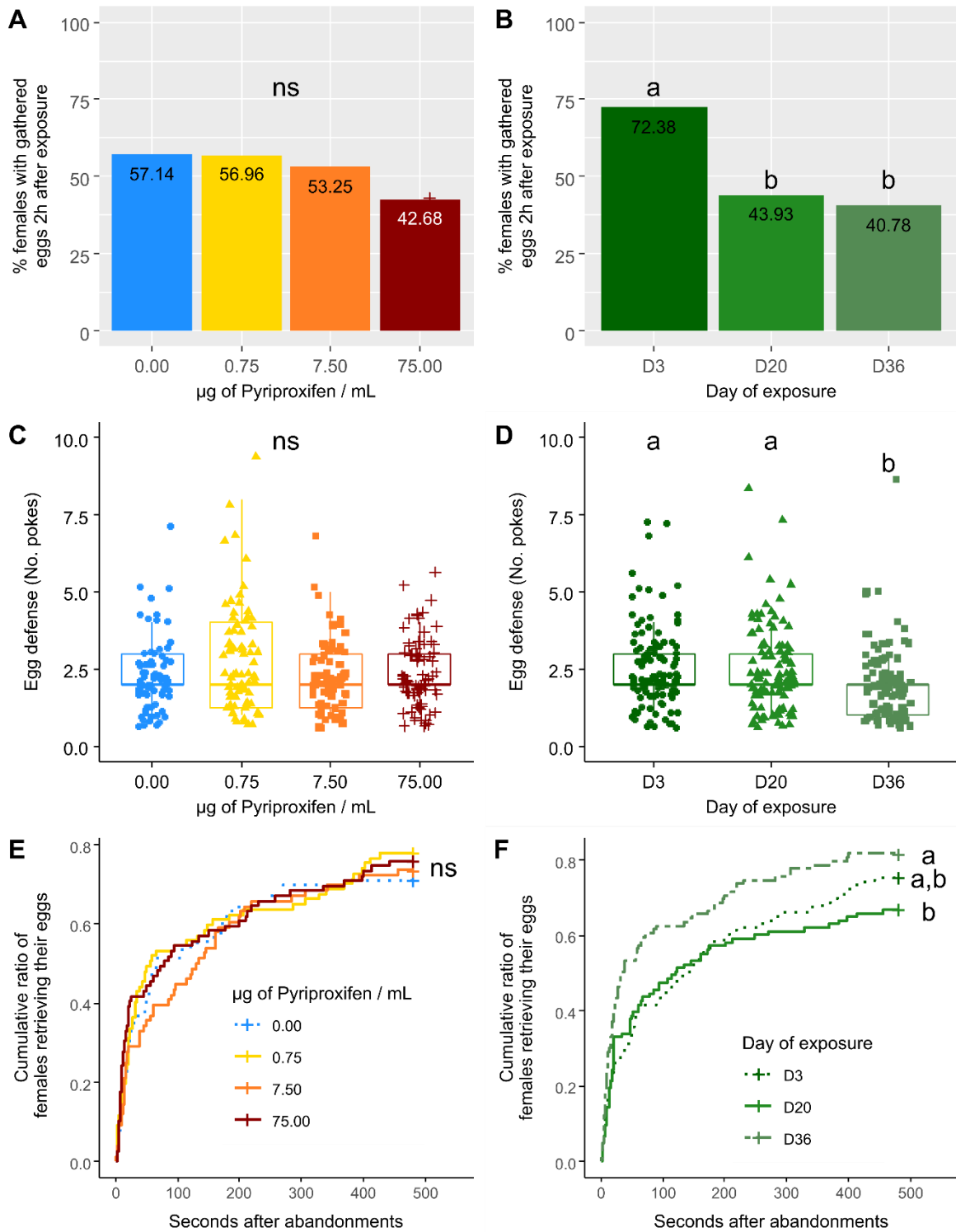
**Table 1** – Statistical effects of pyriproxyfen exposure (A) before and (B) after egg hatching. The day of measurement also refers to the day of pyriproxyfen exposure, as the two are closely related and only 0-2 days apart. P-values below 0.05 are in bold.

	Day of measurement	Pyriproxyfen concentrations			Day of measurement			Interaction		
		Statistics	df	P-value	Statistics	df	P-value	Statistics	df	P-value
<b>(A) Female exposure before egg hatching</b>										
Egg gathering	2h after exposure	4.71	3	0.1944	26.42	2	<b>0.0000</b>	9.31	6	0.1568
Frequency of mother-eggs contacts	2d after exposure	0.62	3	0.6171	0.18	2	0.1825	0.84	6	0.8426
Egg defense	2d after exposure	0.29	3	0.2884	0.00	2	<b>0.0022</b>	0.30	6	0.3043
Egg retrieval time	2d after exposure	0.85	3	0.8379	7.53	2	<b>0.0232</b>	7.61	6	0.2679
Egg gathering	5d after exposure	3.10	3	0.3769	1.41	2	0.4932	9.43	6	0.1507
Mother-egg distance	5d after exposure	0.58	3	0.5841	0.25	2	0.2466	0.18	6	0.1780
Resting frequency	2d after exposure	0.05	3,306	0.9851	3.87	2,306	<b>0.0218</b>	0.31	6,306	0.9336
Self-grooming frequency	2d after exposure	0.73	3	0.8652	16.84	2	<b>0.0002</b>	5.47	6	0.4853
Mother general activity	2d after exposure	1.45	3,293	0.2292	9.39	2,293	<b>0.0001</b>	0.83	6,293	0.5462
Proteins per mg of mothers	Egg hatching	2.15	3,111	0.0977	1.45	2,111	0.2393	0.72	6,111	0.6342
Lipids per mg of mothers	Egg hatching	0.86	3,111	0.4622	0.35	2,111	0.7083	0.84	6,111	0.5406
Glycogen per mg of mothers	Egg hatching	0.63	3,111	0.5964	0.75	2,111	0.4743	0.72	6,111	0.6320
No. nymphs produced	Egg hatching	2.15	3,224	0.0945	0.03	2,224	0.9657	0.53	6,224	0.7874
Mean nymph weight	Egg hatching	0.48	3,219	0.6963	1.99	2,219	0.1389	0.15	6,219	0.9885
<b>(B) Female exposure after egg hatching</b>										
Family gathering	1h after exposure	0.61	3	0.8947	8.47	1	<b>0.0036</b>	2.56	3	0.4643
Frequency of mother-nymphs contacts	2d after exposure	2.70	3,99	0.0500	8.01	1,99	<b>0.0056</b>	1.24	3,99	0.2982
Nymph defense	2d after exposure	0.12	3,99	0.9474	0.08	1,99	0.7725	0.32	3,99	0.8141
Maternal food provisioning	3d after exposure	1.76	3	0.6242	2.62	1	0.1056	3.65	3	0.3019
Resting frequency	2d after exposure	2.31	3,99	0.0807	5.67	1,99	<b>0.0191</b>	0.54	3,99	0.6547
Self-grooming frequency	2d after exposure	0.63	3,99	0.5997	10.73	1,99	<b>0.0015</b>	1.17	3,99	0.3238
Mother general activity	2d after exposure	1.14	3,94	0.3388	0.11	1,94	0.7455	2.12	3,94	0.1030
Nymph survival until day 13	End of family life	1.61	3	0.6566	0.00	1	0.9593	4.69	3	0.1959
Production of a 2nd clutch	End of family life	1.75	3	0.6259	1.24	1	0.2662	2.60	3	0.4582
Delay until 2nd clutch production	End of family life	1.74	3,37	0.1750	0.59	1,37	0.4492	0.21	3,37	0.8919
No eggs in the 2nd clutch	End of family life	0.89	3,36	0.4544	0.35	1,36	0.5572	0.34	3,36	0.7940

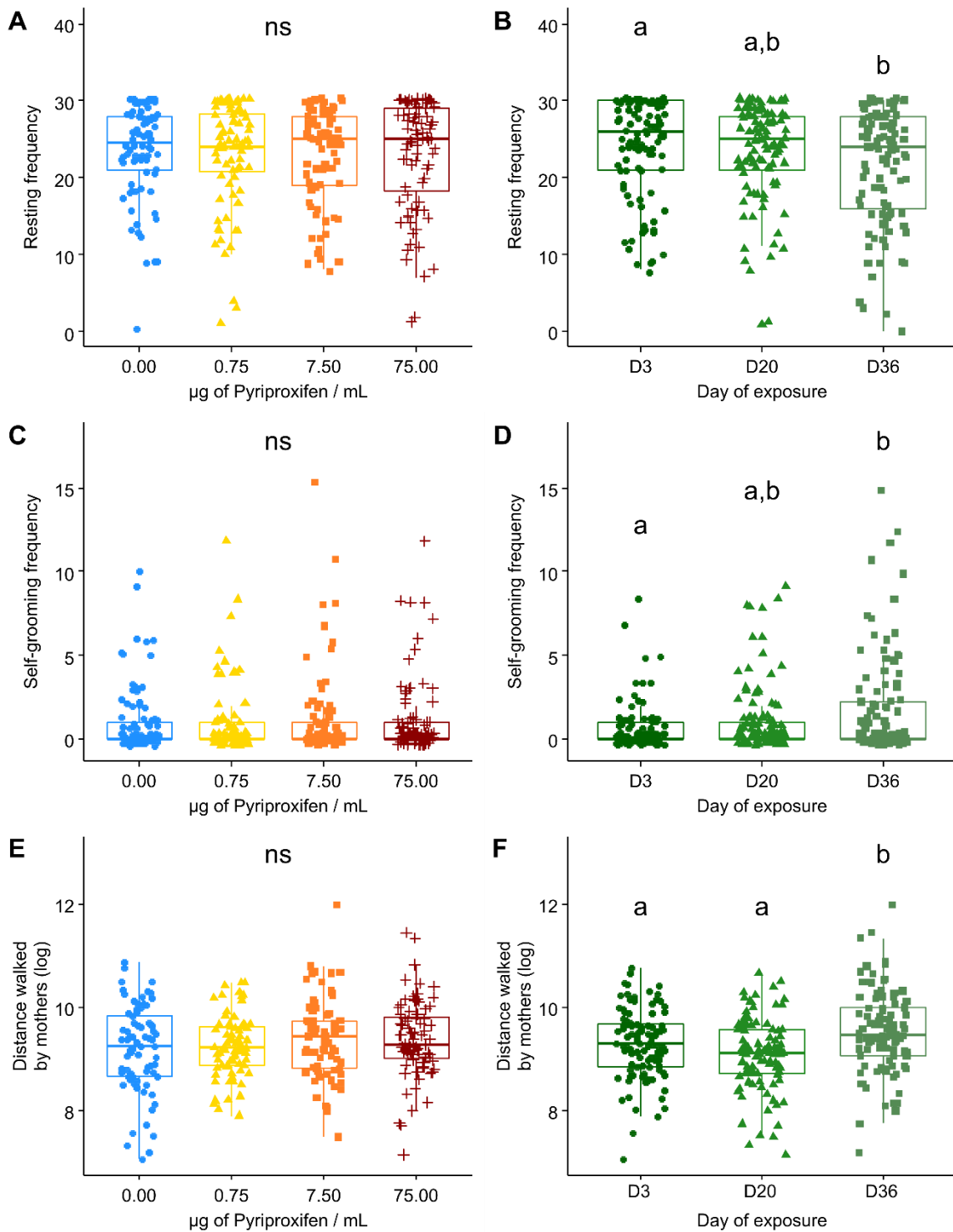
**Table 2** – Descriptive statistics on the effects of pyriproxyfen on 12 parameters measured in earwig mothers and juveniles. SE = Standard Errors.

	Pyriproxyfen concentration ( $\mu\text{g/mL}$ )							
	0.00		0.75		7.50		75.00	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
No. mother-eggs contacts at day 2	2.52	0.32	3.11	0.37	2.88	0.39	2.55	0.40
Mother-eggs distance at day 5 (mm)	4.77	0.48	4.83	0.46	5.33	0.52	4.38	0.49
Proteins ( $\mu\text{g/mg}$ )	5.34	0.30	5.92	0.37	4.94	0.17	5.21	0.20
Lipids ( $\mu\text{g/mg}$ )	13.93	1.31	15.32	1.84	12.16	1.35	14.45	0.95
Glycogen ( $\mu\text{g/mg}$ )	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
No. Nymphs at hatching	10.47	0.94	11.74	1.01	9.99	0.93	10.35	0.90
Nymph weight at hatching (mg)	1.56	0.03	1.58	0.04	1.54	0.04	1.60	0.04
Nymph defense (No. Pokes)	2.93	0.59	2.69	0.26	2.52	0.43	5.17	2.11
Nymph survival at the end of family life (%)	0.82	0.04	0.75	0.05	0.78	0.04	0.04	0.77
Second clutch production (%)	33.33	-	38.46	-	40.00	-	51.72	-
Delay until 2nd clutch production (days)	29.56	2.02	35.18	1.98	35.60	2.03	35.53	2.02
No. 2nd clutch eggs	6.63	1.96	4.92	1.70	5.68	1.89	7.97	1.85

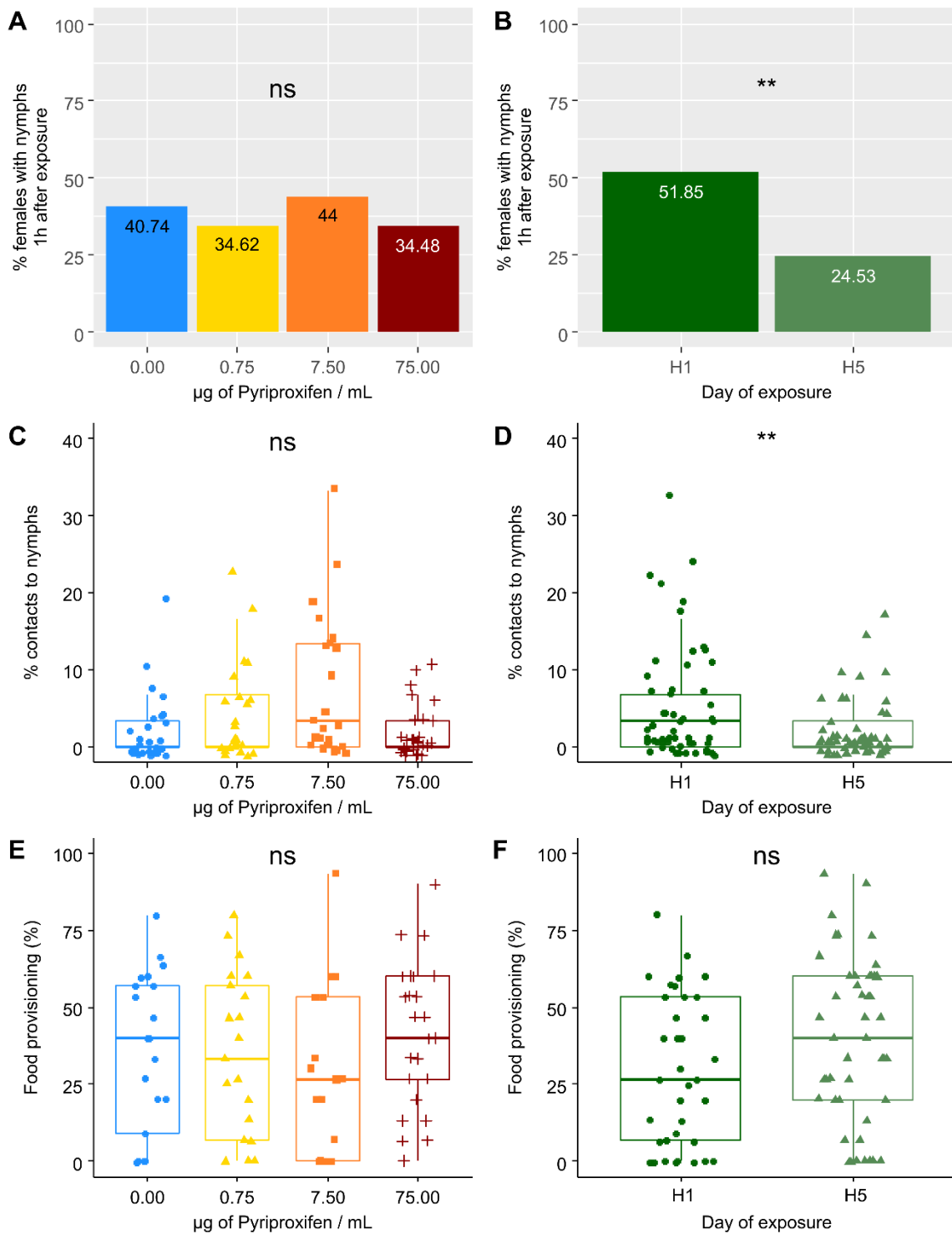
**FIGURE LEGENDS**



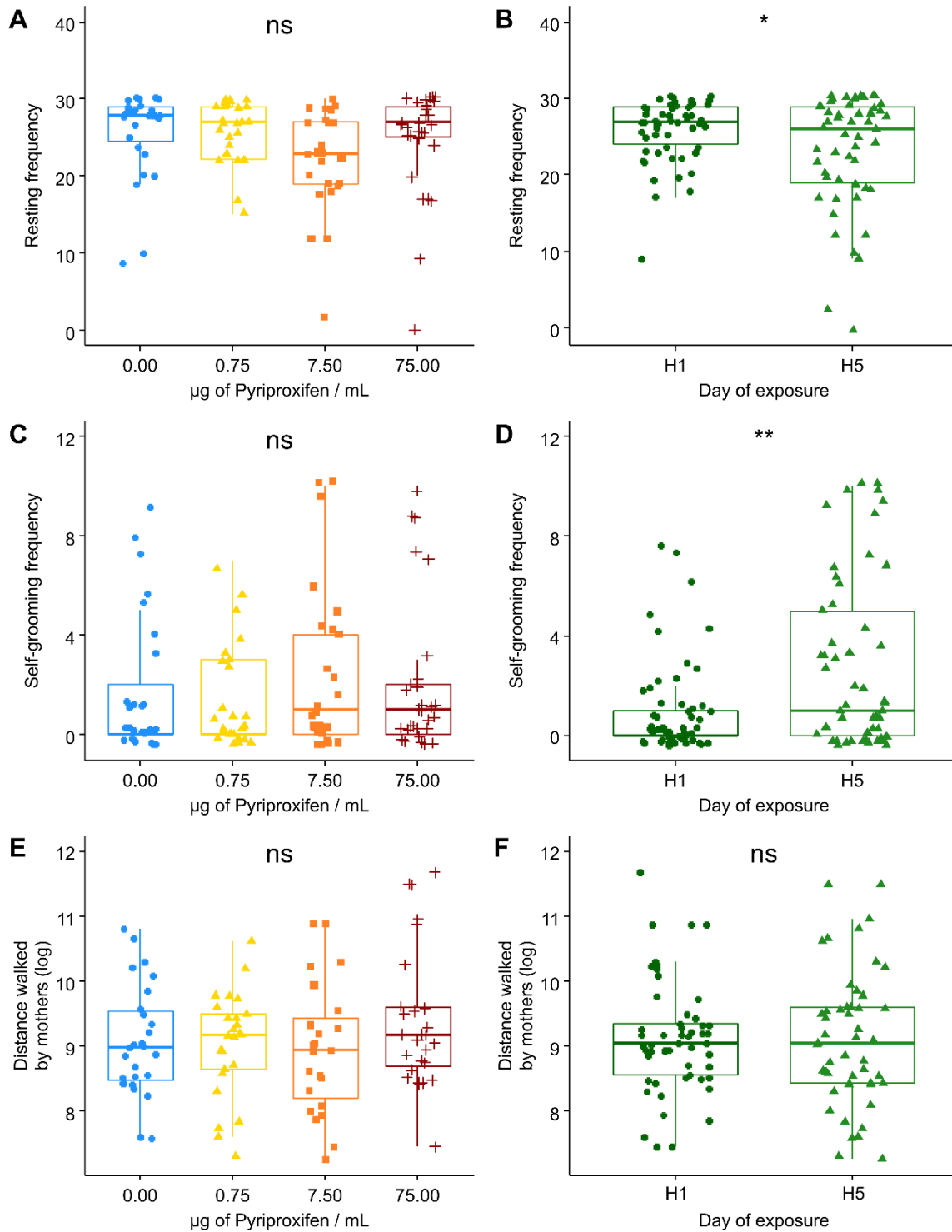
**Figure 1** – Overall effects of pyriproxyfen exposure (left) and day of measurement (right) before egg hatching on the expression of three forms of maternal egg care. (A, B) The likelihood that mothers would collect their experimentally dispersed eggs 2h after exposure. (C, D) Level of egg defence against a simulated predator attack 2 days after exposure. (E, F) The speed at which females retrieved their eggs after this simulated predator attack. The day of exposure is provided as a standard indication of the day of measurement. Boxplots depict median and interquartile range, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. Different letters refer to  $p < 0.05$ . <sup>ns</sup>  $p > 0.05$ .



**Figure 2** – Overall effects of pyriproxyfen exposure (left) and day of measurement (right) before egg hatching on the expression of three non-care behaviours by females. (A, B) Resting frequency of mothers 2 days after exposure. (C, D) The Self-grooming frequency 2 days after exposure. (E, F) General activity of mothers in absence of eggs measured via the distance walked during 30 min. The day of exposure is provided as a standard indication of the day of measurement. Boxplots depict median and interquartile range, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. Different letters refer to  $p < 0.05$ .  $^{ns}$   $p > 0.05$ .



**Figure 3** – Overall effects of pyriproxyfen exposure (left) and day of measurement (right) after egg hatching on the expression of three forms of maternal egg care. (A, B) The likelihood that mothers would return to their nymphs 1h after exposure. (C, D) The proportion of mother-offspring contacts 2 days after exposure. (E, F) The proportion of nymphs receiving food from their mother 4 days after exposure. The day of exposure is provided as a standard indication of the day of measurement. Boxplots depict median and interquartile range, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. Different letters refer to  $p < 0.05$ . *ns*  $p > 0.05$ .



**Figure 4** - Overall effects of pyriproxyfen exposure (left) and day of measurement (right) after egg hatching on the expression of three non-care behaviours by females. (A, B) Resting frequency of mothers 2 days after exposure. (C, D) Self-grooming frequency 2 days after exposure. (E, F) General activity of mothers in absence of nymphs measured via the distance walked during 30 min. The day of exposure is provided as a standard indication of the day of measurement. Boxplots depict median and interquartile range, with whiskers extending to 1.5 times the interquartile range and dots representing jittered experimental values. \*\*  $p < 0.01$ ; \*  $p < 0.05$ ; <sup>ns</sup>  $p > 0.05$ .