

SHORT COMMUNICATION

Do arthropods feel anxious during molts?

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ABSTRACT

The molting process of arthropods, chiefly controlled by ecdysteroids, is generally considered very stressful. Our previous investigations have shown that crayfish, after having experienced stressful situations, display anxiety-like behavior (ALB), characterized by aversion to light in a dark/light plus-maze (DLPM). In the present experiments, the spontaneous exploratory behavior of isolated crayfish was analyzed in a DLPM at different stages of their molt cycle. All tested animals displayed transitory aversion to light similar to ALB, before and, mostly, after molting, but not during inter-molt. Injection of ecdysteroids into inter-molt animals elicited ALB after a delay of 4 days, suggesting a long-term, possibly indirect, hormonal effect. Importantly, ecdysteroid-induced ALB was suppressed by the injection of an anxiolytic benzodiazepine. Thus, molts and their hormonal control impose internal stress on crayfish, leading to aversion behavior that has the main characteristics of anxiety. These observations are possibly generalizable to many other arthropods.

KEY WORDS: Anxiety, Stress, Plus-maze, Ecdysteroids, Crayfish

INTRODUCTION

Arthropods are characterized by a tegument covered with an articulated cuticle exoskeleton that is essential for protection against external aggression and for locomotion. This cuticle also supports many sensory organs and is involved in the control of vital exchanges, such as respiration, control of water content, feeding and excretion (Wigglesworth, 1972). However, this rigid carapace limits body growth and prevents changes in body form. Thus, it must be renewed periodically to allow growth or body transformation. This overall process, generally called molt or molting, is not limited to the rapid behavior of shedding the old shell, which is more precisely named ecdysis, but involves a long cascade of events that precede and follow ecdysis (Jenkin and Hinton, 1966; Wigglesworth, 1972). The pre-ecdysial period (here, pre-molt) starts with the progressive separation of the cuticle from the epidermis (apolysis) and the partial digestion of the inner layers of the old cuticle. It also includes the proliferation of epidermal cells, the beginning of the secretion of a new cuticle under the old one, the transfer of muscle attachments to the new cuticle, and the reorganization of all associated sensory

organs. At ecdysis, a stereotyped behavior leads to the rapid shedding of the old carapace, revealing the thin and fragile new cuticle, which is progressively thickened and hardened during the following post-ecdysial period (here, post-molt). Animals become highly vulnerable during molts, particularly after ecdysis. Therefore, they frequently seek safe places to hide during pre-molt, where they remain inert until post-molt (Wigglesworth, 1972). These molt events can be considered very stressful, which raises the question of whether arthropods fear molting.

Our previous observations (Fossat et al., 2014, 2015a,b; Bacqué-Cazenave et al., 2017) have demonstrated that crayfish respond to stressful situations, such as physical or social stress, by displaying anxiety-like behavior (ALB) that can be measured using an aquatic dark/light plus-maze (DLPM). The first aim of the present study was to determine whether the stressful events of molts induce anxiety-like reactions in crayfish.

In vertebrates, the control of anxiety frequently involves steroid hormones and neurosteroids, which may have either anxiogenic or anxiolytic properties (Korte, 2001; Dubrovsky, 2005; Holsboer and Ising, 2010). Though vertebrate steroids can be detected in arthropods (Ollevier et al., 1986) and may have some physiological effects (Snyder and Peeke, 2001), they probably have only an alimentary origin. Ecdysteroids are the only endogenous steroid hormones synthesized in several organs of these animals (Lafont, 2000), but not in the nervous system (Warren et al., 1999). Also known as molting hormones, ecdysteroids essentially induce apolysis and other pre-molt events, but also play a role in many aspects of arthropod physiology, reproduction and behavior. In particular, small concentrations of ecdysteroids initiate the wandering behavior of many holometabolous insects, allowing them to find convenient places to pupate, whereas higher concentrations inhibit locomotion and lead to the quiescent state observed before ecdysis (Dominick and Truman, 1985). Ecdysis is also inhibited by ecdysteroids (Krishnakumaran and Schneiderman, 1970; Maissiat and Graf, 1973; Slamà, 1980) and occurs only after the concentration of ecdysteroids returns to basal levels (Truman, 2005). Other studies have emphasized that ecdysteroid concentrations are reduced after stress (Mattson and Spaziani, 1985), suggesting that these steroids participate in the control of stress responses during molts. Therefore, the second aim of this study was to analyze whether ecdysteroids could control behavior in terms of anxiety-like reactions.

MATERIALS AND METHODS

Animals

Crayfish, *Procambarus clarkii* (Girard 1852), were collected from swamps near Bordeaux (Réserve Naturelle de Bruges) and stored next to the laboratory inside an animal house at 20°C under a 12 h:12 h light:dark cycle. The animals were fed pellets regularly. Males weighing between 20 and 40 g were marked by a dot painted on the thorax (to facilitate detection of cuticle change) and isolated in individual tanks (50×30×30 cm, equipped with recirculating water). In our laboratory conditions, mature males continued to grow after each molt.

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To avoid stressing the animals, we did not evaluate the individual progression in molting cycles of crayfish by repeated observations of swimmerets or by hemolymph sampling for ecdysteroid measurements. However, all tanks were observed every 1 or 2 days, and ecdysis occurrence was noted for each animal; the behavioral observations, which were made earlier or later, were dated in weeks or days before or after ecdysis. The inter-molt period, during which animals are not engaged in molting, is long in adult crayfish, even under controlled conditions; in our laboratory, this period lasted 2–6 months. By contrast, ecdysis is a very short sequence lasting only a few minutes, which is difficult to predict and observe (see Movie 1). However, the whole process of molting takes much more time than does ecdysis. In our experimental conditions, we estimated that pre-molt lasted approximately 2 weeks, which we subdivided into two equal parts: early pre-molt (14–7 days before ecdysis) and late pre-molt (6–0 days before ecdysis). The period following ecdysis was subdivided into early post-molt (0–2 days following ecdysis) and late post-molt (3–14 days after ecdysis). Early post-molt was the most easily observable period, marked by the newly molted animal's presence near its old shell, which was generally consumed 2–3 days after ecdysis.

Behavior analysis

We used the aquatic DLPM protocol described earlier (Fossat et al., 2014, 2015b). Briefly, the DLPM (60×60 cm) comprised two dark arms (light intensity: 10 lx; 25 cm length, 10 cm width) and two illuminated (light) arms (light intensity: 50 lx; same dimensions as the dark arms). Each tested crayfish was placed in DLPM center for 1 min under an opaque cover. After lifting the cover, exploratory behavior was recorded from above with a video camera (Sony Inc., Tokyo, Japan). In contrast to our previous studies in which animals were submitted to a stress and tested only once in DLPM, the present experiments were made on unstressed isolated males, tested regularly in DLPM, generally every 2–3 days during the natural molt cycle or every day after hormonal treatment (see below). We used Ethovision software XT8 (Noldus, Wageningen, The Netherlands) to detect and track crayfish in the arena. Seven behavioral variables were recorded as previously described (Fossat et al., 2014). However, we focused particularly on the results of two variables: the percentage of time spent in light areas and the retreat ratio (calculated as the ratio between the number of retreats and the total number of attempts to enter a light area).

Injections

20-Hydroxyecdysone (20E; Sigma Aldrich, St Louis, MO, USA) was administered as previously described (Bacqué-Cazenave et al., 2013). A single dose was injected intramuscularly to obtain a final concentration of 5 µg 20E g⁻¹ fresh mass. Lower doses (0.5 µg g⁻¹) and saline injections were administered similarly to other animals.

The anxiolytic benzodiazepine chlordiazepoxide (CDZ; Sigma Aldrich) was injected intramuscularly in crayfish previously injected with 20E. CDZ was administered at a final concentration of 15 µg g⁻¹, according to our previous observations (Fossat et al., 2014).

Statistical analyses

Means (±s.e.m.) of each behavioral variable in the different experimental groups were compared with Friedman tests, followed by *post hoc* analysis (Dunn's multiple comparison tests). A comparison of groups with all their behavioral variables was also performed using multivariate analysis of variance (MANOVA) and principal component analysis (PCA) using R software. PCA was performed as previously described (Fossat et al., 2014). After PCA,

the separation between pairs of groups was evaluated by calculating the inertia, which was defined as the ratio of the between-group variance to the global variance. The statistical significance of inertia for group separation was estimated using a Monte Carlo permutation test (1000 runs) and evaluated at $P < 0.01$. In each run, the simulated inertia was calculated. The distribution of simulated inertia values was then compared with the real inertia. A P -value was subsequently calculated as the ratio of the number of simulations in which the simulated inertia was larger than the real inertia to the total number of runs.

RESULTS AND DISCUSSION

Anxiety-like behavior during molt

Exploratory behavior in the DLPM was regularly observed, according to the previously described protocol (Fossat et al., 2014, 2015b), for isolated males, throughout the molt/inter-molt cycle. Despite the repetition of such analyses, the behavior of unstressed animals remained constant not only in inter-molt but also in early pre-molt, during which they spent similar percentages of time exploring the illuminated (light) arms of the DLPM (Fig. 1Ai; Fig. S1). We did not make observations during ecdysis, as this short and stereotyped sequence was incompatible with the DLPM protocol. However, all observations performed a few hours after ecdysis and during early post-molt revealed statistically significant changes (Friedman tests) in exploratory behavior from that observed during pre-molt, including a decrease in the amount of time spent in the light arms (Fig. 1Ai) and an increase in retreats when entering the illuminated areas (Fig. 1Aii). The highly significant change in behavior during early post-molt was preceded during late pre-molt by a trend that was not significant when the variables were analyzed separately. However, the use of PCA allowed us to demonstrate that, considering the changes observed for all seven uncorrelated behavioral variables (Fig. 1Bi; Fig. S1), the animals expressed low but significant avoidance behavior during late pre-molt (Fig. 1Bii, iii). MANOVA also confirmed that the relative time spent in the light and the retreat ratio were significantly modified ($P = 0.0005$ and $P = 0.0002$, respectively), but not locomotion ($P = 0.8$).

These molt-related changes in the exploratory behavior of crayfish, particularly increased avoidance of illuminated areas of the DLPM and increased retreats, were similar to those previously observed for crayfish exposed to either physical stress (Fossat et al., 2014) or social stress (Bacqué-Cazenave et al., 2017), which were interpreted as ALB. In the present experiments with animals that were not subjected to external stress, behavior with the features of ALB was observed spontaneously during the molt cycle, particularly after ecdysis. This behavior was also observed at a lower intensity a few days before ecdysis, suggesting that it was not only an immediate consequence of ecdysis but also the result of an internal stress that gradually increased before ecdysis, probably because of hormonal modification. Such observations are reminiscent of stress and anxiety disorders observed in mammals, including humans, in relation to the drastic hormonal changes that occur during development, such as puberty, pregnancy or aging (Ross and McLean, 2006; Leonardo and Hen, 2007; Reardon et al., 2009; Domonkos et al., 2017).

Effects of ecdysteroids

In crustaceans, as in most other arthropods, 20E (formerly also known as crustecdysone) is the main ecdysteroid (Horn et al., 1968). Injections of high amounts of exogenous 20E into arthropods are generally able to induce pre-molt but not the entire molting cascade comprising ecdysis and post-molt events, whereas lower

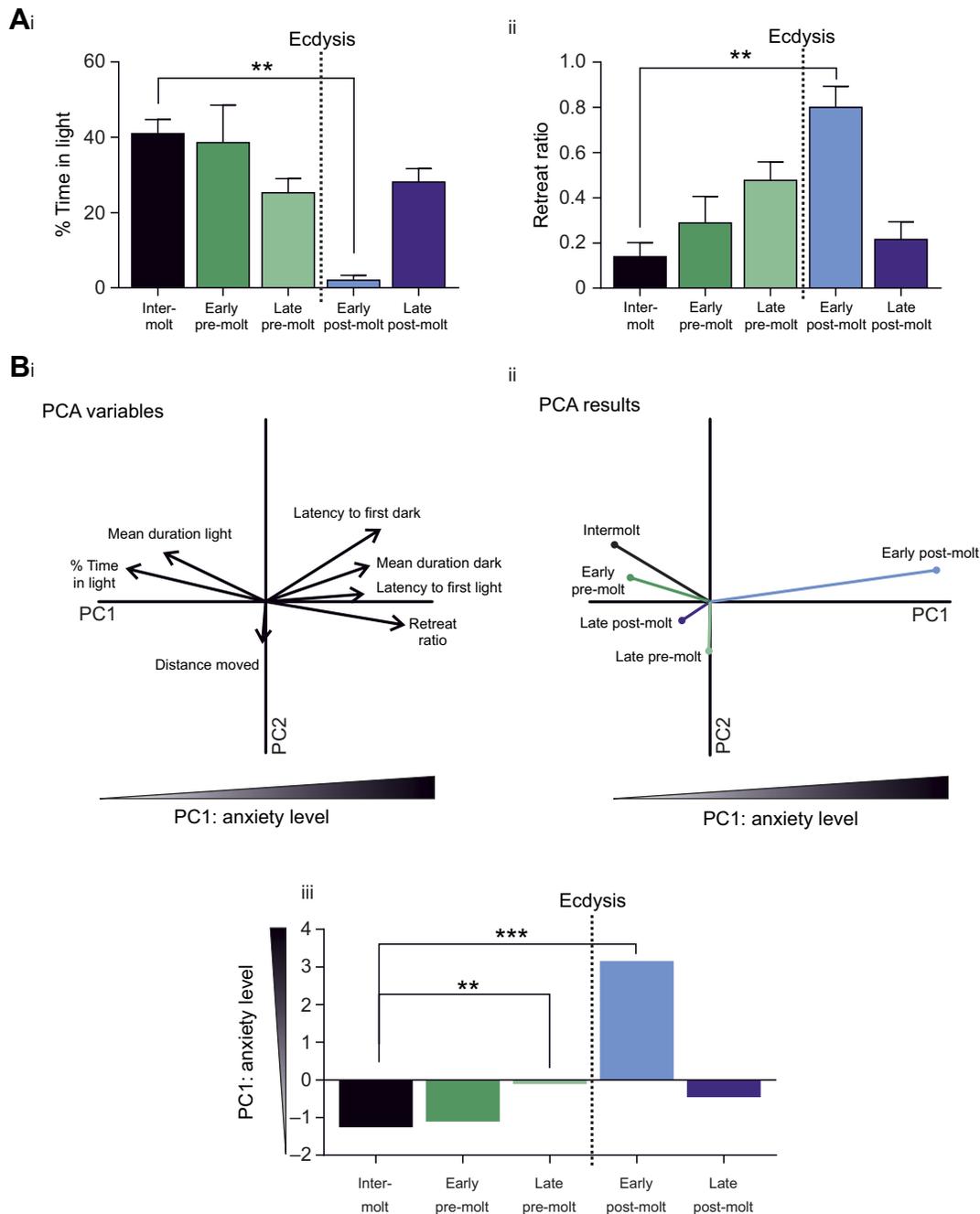
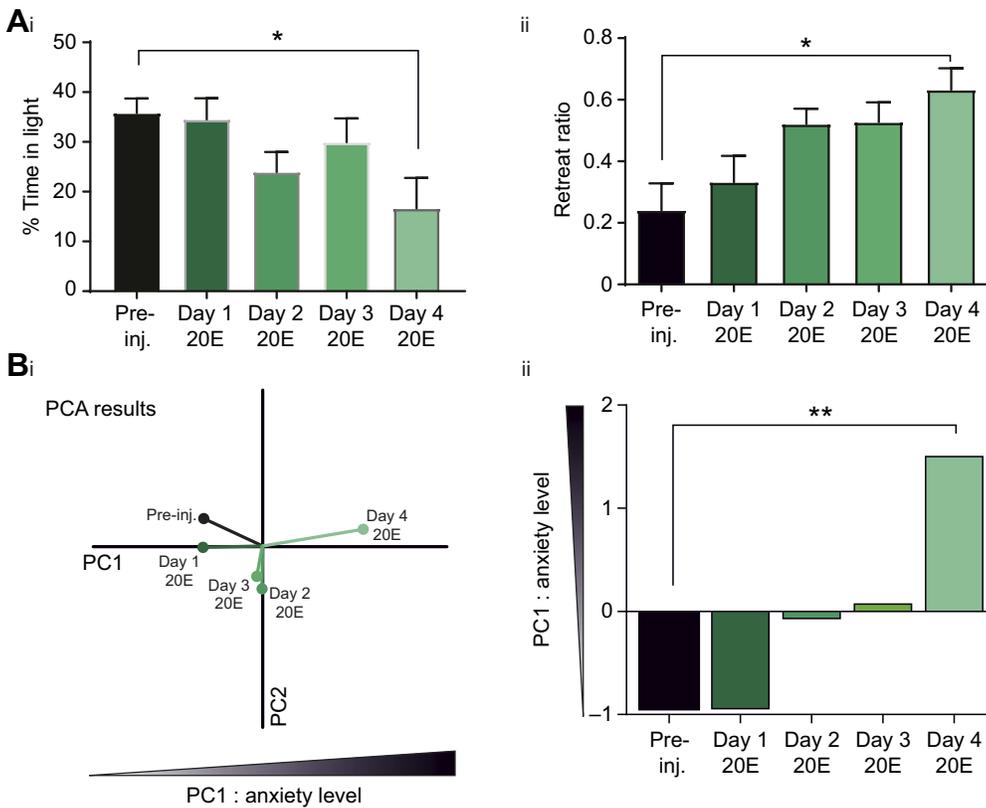


Fig. 1. Anxiety-like behavior during molt. Crayfish ($n=6$) were tested individually every 2–3 days in a dark/light plus-maze (DLPM). Observations were gathered into the 5 different substages along the molting cycle: inter-molt (more than 14 days after/before ecdysis), early pre-molt (14–7 days before ecdysis), late pre-molt (6–0 days before ecdysis), early post-molt (0–2 days after ecdysis) and late post-molt (3–14 days after ecdysis). (A) DLPM analysis: the percentage of time spent in the light arms (Ai) was altered during the molt cycle. However, mean values did not significantly differ between inter-molt and early pre-molt ($41.0 \pm 3.7\%$ versus $38.6 \pm 9.9\%$, $P > 0.05$, Friedman test followed by Dunn's multiple comparison test) or late pre-molt ($25.3 \pm 3.7\%$, $P > 0.05$, Friedman test followed by Dunn's multiple comparison test), but a significant decrease was observed in early post-molt ($2.1 \pm 1.3\%$, $**P < 0.01$, Friedman test followed by Dunn's multiple comparison test). In late post-molt, the percentage ($28.1 \pm 3.6\%$, $P > 0.05$, Friedman test followed by Dunn's multiple comparison test) was not significantly different from that in inter-molt. The retreat ratio (Aii) showed non-significant increases from inter-molt (0.14 ± 0.06) to the pre-molt periods (early: 0.29 ± 0.12 ; late: 0.48 ± 0.08). It became significantly different from inter-molt in the early post-molt period (0.80 ± 0.09 , $**P < 0.01$, Friedman test followed by Dunn's multiple comparison test). It then decreased in late post-molt (0.21 ± 0.08) to a level similar to that in inter-molt ($P > 0.05$). (B) Principal component analysis (PCA): the contributions of the seven uncorrelated variables (Bi) are represented on two PCA axes: the x-axis represents the first component (PC1), interpreted as anxiety level, and the y-axis represents the second component (PC2), interpreted as mobility (see Fossat et al., 2014). The barycenters of the different molt periods are represented on the PCA (Bii). To better visualize relative anxiety level, the results of comparisons between groups are shown in a scheme (Biii) representing only the first component values. The comparisons showed significant differences between the late pre-molt and early post-molt groups and the inter-molt group ($**P < 0.01$ and $***P < 0.001$, respectively, Monte-Carlo tests).

**Fig. 2. Effects of ecdysteroids.**

(A) DLPM analysis ($n=7$): inter-molt animals were subjected to DLPM analysis before and after injection with 20-hydroxyecdysone (20E; $5 \mu\text{g g}^{-1}$). The results (Ai) showed that the percentage of time spent in the light arms during the first 3 days (1st day: $34.5 \pm 4.2\%$; 2nd day: $23.9 \pm 4\%$; 3rd day: $29.9 \pm 4.8\%$) did not significantly differ from that before injection ($36 \pm 3.0\%$, $n=7$, $P>0.05$, Friedman test followed by Dunn's test), but decreased significantly on the 4th day ($16.6 \pm 6\%$, $*P<0.05$). The retreat ratio (Aii) progressively increased over the 3 days after injection (1st day: 0.33 ± 0.09 ; 2nd day: 0.52 ± 0.05 ; 3rd day: 0.53 ± 0.06) and became significantly different on the 4th day (0.63 ± 0.07 , $*P<0.05$ Dunn's test). (B) PCA: the results of the PCA (Bi) and the group comparisons (Bii) confirmed that the behavior on the 4th day after injection was significantly different from that before injection ($**P<0.01$, Monte-Carlo test) and that the behavior 2–3 days after injection was intermediate between pre-injection and day 4 after injection.

doses are inefficient at inducing pre-molt (Krishnakumaran and Schneiderman, 1970). In agreement with these observations and with a previous study (Bacqué-Cazenave et al., 2013), the injection of 20E at a high dose ($5 \mu\text{g g}^{-1}$) into crayfish triggered pre-molt but not ecdysis, whereas injections of saline or a lower dose of 20E ($0.5 \mu\text{g g}^{-1}$) did not induce pre-molt. The failure of ecdysis observed after high-dose injection was ultimately deleterious, causing the animals to die 7–10 days after injection, probably by suffocation. However, as observed previously (Bacqué-Cazenave et al., 2013), the animals remained in good health for more than 5 days and the observations made during this period are thus worth considering as a model of pre-molt induction.

Isolated crayfish were tested in the DLPM before being injected with $5 \mu\text{g g}^{-1}$ 20E and again after injection as well as during the 4 days following injection, i.e. long before the deleterious effects of ecdysis impairment. Fig. 2A shows that animals displayed changes in behavior similar to ALB 4 days after the hormonal treatment (Friedman test). MANOVA also showed that retreat ratios were significantly different between groups ($P=0.003$), but that locomotion did not significantly change ($P=0.7$; see also Fig. S2A). PCA analysis (Fig. 2B; Fig. S2) revealed a trend toward ALB from the second day after injection, which was significant only on the fourth day. By contrast, isolated animals injected with either saline or $0.5 \mu\text{g g}^{-1}$ 20E did not display ALB (data not shown).

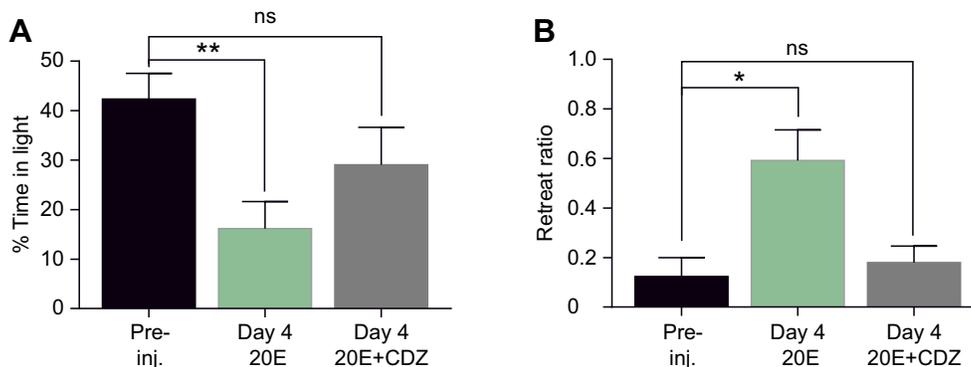


Fig. 3. Suppression of anxiety-like behavior (ALB) by a benzodiazepine. Effects of injection of chlordiazepoxide (CDZ) on (A) the percentage of time spent in light areas and (B) the retreat ratio, observed 4 days after 20E injection. Animals ($n=5$) were successively subjected to DLPM trials before 20E injection (Pre-inj.), 4 days after 20E injection (Day 4 20E) and 30 min after CDZ injection (Day 4 20E+CDZ), which was performed 1 h after the DLPM test for Day 4 20E. In agreement with Fig. 2, 20E treatment significantly affected the percentage of time spent in the light arms (Pre-inj.: $42.23 \pm 5.20\%$; Day 4 20E: $16.18 \pm 5.44\%$, Dunn's test, $**P<0.01$) and the retreat ratio (Pre-inj.: 0.12 ± 0.08 ; Day 4 20E: 0.59 ± 0.12 , Dunn's test, $*P<0.05$). However, CDZ injection reversed the effect of 20E on the percentage of time spent in the light area and on the retreat ratio (Friedman test: $P<0.01$ and $P<0.05$, respectively): there were no significant differences (Dunn's tests, ns) between pre-injection and 20E+CDZ-treated crayfish in the percentage of time spent in the light arms (Day 4 20E+CDZ: $29.05 \pm 7.60\%$) and the retreat ratio (Day 4 20E+CDZ: 0.18 ± 0.07).

These observations suggest that 20E is able to modulate ALB. However, 20E probably does not act on ALB through a rapid and direct effect but through a delayed and possibly indirect effect. This hypothesis may explain why low doses of 20E are inefficient, as well as why the first signs of ALB appeared during the natural molting cycle in late pre-molt but not early pre-molt. Numerous studies have shown that ecdysteroid titers increase progressively during early pre-molt and decrease in late pre-molt (Stevenson et al., 1979; Nakatsuji and Sonobe, 2004) and that the mode of action of ecdysteroids involves complex signaling cascades that control sequential events, such as the secretion of cuticle layers and ecdysis: early events are initiated by an increase in ecdysteroids, but the decrease in hormonal titers at the end of pre-molt is necessary for the completion of cuticle secretion, ecdysis and tanning, which may also involve intermediary peptide hormones (Truman, 2005; Žitňan et al., 2007; White and Ewer, 2014). Although we have not analyzed the kinetics of ecdysteroids after 20E injection, we can assume that our experiments partly mimic both the increase and the decrease of ecdysteroid titers during pre-molt: indeed, the injection of ecdysteroids into crustaceans is followed by the rapid elimination of exogenous hormones and their metabolites into urine (Snyder and Chang, 1991). Thus, a control of ALB after a decrease in ecdysteroid titers can be hypothesized, and whether peptides or other hormones could be intermediate hormonal regulators of ALB remains an open question.

Suppression of ALB by a benzodiazepine

Our previous experiments have established that anxiolytic drugs such as CDZ can suppress stress-related ALB (Fossat et al., 2014). In the following experiment, we aimed to check whether hormone-related ALB was also sensitive to CDZ. Four days after treatment with $5 \mu\text{g g}^{-1}$ 20E and 1 h after DPLM observation, some animals were injected with $15 \mu\text{g g}^{-1}$ CDZ, and subjected to a DLPM trial 30 min later. The results (Fig. 3) showed that the steroid-induced ALB was rapidly suppressed after CDZ treatment. These data confirmed that the changes in behavior induced by ecdysteroids were really related to anxiety. They also confirmed that 20E-related ALB was not due to a deleterious effect of the hormonal injection.

In conclusion, crayfish molt involves a transitory light-aversion behavior that can be related to anxiety, because of its sensitivity to benzodiazepines, and that is controlled by ecdysteroids through a long-term effect. These observations are probably generalizable to many arthropods.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.P.D., D.C., P.F., J.B.-C.; Methodology: D.C., P.F., J.P.D.; Formal analysis: J.B.-C., P.F.; Investigation: J.B.-C., M.B., P.F., J.P.D.; Writing - original draft: J.P.D.; Writing - review & editing: D.C., P.F., J.P.D., J.B.-C.; Supervision: P.F., J.P.D.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.186999.supplemental>

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