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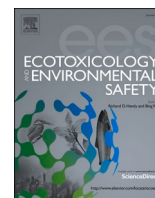
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# The retinoid metabolism of *Gammarus fossarum* is disrupted by exogenous all-trans retinoic acid, citral, and methoprene but not by the technical formulation of glyphosate

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## ABSTRACT

Over the last decade, fluctuations of retinoids (RETs), also known as vitamin A and derivatives, have proved to be useful biomarkers to assess the environmental chemical pressure on a wide variety of non-target vertebrates. This use of RET-based biomarkers is of particular interest in the non-target sentinel species *Gammarus fossarum* in which RETs were shown to influence crucial physiological functions. To study and probe this metabolism in this crustacean model, a UHPLC-MS/MS method was developed to 1) identify and 2) monitor several endogenous RETs in unexposed females throughout their reproductive cycle. Then, females were exposed in controlled conditions to exogenous all-trans retinoic acid (atRA) and citral (CIT), a RA synthesis inhibitor, to simulate an excess or deficiency in RA. Perturbation of vitamin A metabolism by pesticides was further studied in response to methoprene (MET), a juvenile hormone analog as well as glyphosate (GLY). The developed method allowed, for the first time in this model, the identification of RA metabolites (all-trans 4-oxo and 13-cis 4-oxo RA), RA isomers (all-trans and 13-cis RA) as well as retinaldehyde (RALD) isomers (all-trans, 11-cis, and 13-cis RALD) and showed two distinct phases in the reproductive cycle. Retinoic acid successfully increased the tissular concentration of both RA isomers and CIT proved to be efficient at perturbing the conversion from RALD to RA. Methoprene perturbed the ratios between RA isomers whereas GLY had no observed effects on the RET system of *G. fossarum* females. We were able to discriminate different dynamics of RET perturbations by morphogens (atRA or CIT) or MET which highlights the plausible mediation of RETs in MET-induced disorders. Ultimately, our study shows that RETs are influenced by exposure to MET and strengthen their potential to assess aquatic ecosystem chemical status.

## 1. Introduction

Retinoids (RETs) are a class of compounds including retinol (vitamin A) and numerous other naturally occurring or synthetic derivatives. Naturally occurring RETs, which are made up of a trimethylated cyclohexene ring, a conjugated tetraene side chain, and a polar carbon-oxygen functional group (Barua and Furr, 1998), are found in a wide variety of vertebrates (mammals, birds, fish, and amphibians) and invertebrates (arthropods, mollusks, and Porifera) (see reviews by Albalat, 2009; André et al., 2014). They are derived from precursors carotenoids

(CARs) or RET esters from the diet and exist in their -trans and -cis molecular configuration. Retinoic acids (RAs), the most biologically active RETs, are essential in many vertebrate cellular processes such as cell growth, differentiation, and apoptosis through the activation of the Retinoid X Receptor (RXR) and the Retinoic Acid Receptor (RAR). For this reason, RAs are crucial for embryonic development, growth, reproduction, and immunity (see review by Kam et al., 2012). Although RET metabolism was thought to be an innovation of chordates for a long time, molecular and biochemical evidence suggested it originated from a common ancestor of all bilaterians (André et al., 2014). Among

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invertebrates, most of the scientific interest was directed toward the benefits of CAR intake in crustacean growth and reproduction to optimize aquaculture (Wade et al., 2015). Still, as of today, despite paving the way for further investigation of the underlying RET metabolism in crustaceans, fundamental knowledge of RET diversity and functions in crustaceans is lacunary. This is especially true for smaller species that are not included in the human diet like amphipods for which no data is available yet.

Fluctuations of hepatic RET concentrations in mammals, birds, fish, and amphibians were reported after laboratory or in-field exposures to a wide variety of xenobiotics notably metallic trace elements (MTEs), pesticides, PCBs, PAHs, dioxins, furans, etc. (Boily et al., 2004; Defo et al., 2014). In many biomonitoring studies, as diminutions of their concentrations were associated with anthropogenic activities, RET isomers in birds, fish, and amphibians proved to be valuable biomarkers (Boily et al., 2004, 2009; Landry et al., 2020). Also, the RET metabolism and signaling in vertebrates have been suggested as a target candidate for endocrine disruption and are considered for future implementation in endocrine-disruptive chemicals (EDCs) toxicity test guidelines (OECD Organisation for Economic Cooperation and Development, 2021). More recently, studies focusing on the honey bee *Apis mellifera* also documented an increase or reduced levels of some RETs after exposure to herbicides, insecticides, or MTEs (Hedrei Helmer et al., 2014; Jumarie et al., 2017; Gauthier et al., 2018). Hedrei Helmer et al., (2014) showed that honey bees exposed to atrazine had a higher concentration of all-*trans* retinol (ROL), while this compound was lower when bees were exposed to metolachlor. In that same study, a tendency to lower ROL content was also recorded following exposure to glyphosate (GLY). In addition, higher levels of RA metabolites (all-*trans* 4-oxo and 13-*cis* 4-oxo RA) were reported when honeybees were exposed to a mixture of atrazine, GLY, and cadmium (Jumarie et al., 2017) or thiamethoxam (Gauthier et al., 2018). The documented usefulness of RET-based biomarkers in assessing the chemical pressure on non-target vertebrates and more recently on the honey bee opens an avenue for such investigations in other arthropod species and portends promising applications in biomonitoring.

*Gammarus fossarum* is an amphipod crustacean that plays a central role in aquatic ecosystems in breaking down organic matter and is of particular importance in food webs for a wide array of predators. Together with cladocerans, gammarids are considered to be among the most sensitive taxa to organic compounds and MTEs and are easy to collect and maintain in laboratory studies (Von der Ohe and Liess, 2004). For these reasons, *G. fossarum* has received much scientific interest as a potential crustacean sentinel to monitor chemical stresses on non-target aquatic organisms in freshwater ecosystems (Kunz et al., 2010; Besse et al., 2013; Chaumot et al., 2015; Alric et al., 2019). In addition, the ovarian maturation, embryogenesis, and molting processes have been thoroughly characterized specifically in this model and were shown to happen concurrently and in a perfectly synchronized manner (Geffard et al., 2010). The desynchronization of these biological processes is an endpoint in the bioassay developed by Geffard et al. (2010) and its monitoring allows the identification of endocrine disruption for crustaceans. In crustaceans, exposure to exogenous RA increases circulating glucose and hormones (methyl farnesoate and ecdysteroids) and perturbs processes under the control of the endocrine system such as reproduction and regeneration (Hopkins, 2001; Reddy and Sainath, 2008; Sreenivasula and Srilatha, 2015; Girish et al., 2018; Venkaiah et al., 2019). However, the physiological implications of RETs as well as their metabolism in crustaceans remain mostly unknown. Recently, we exposed female *G. fossarum* to atRA, citral (CIT), an inhibitor of RA synthesis, and methoprene (MET), a juvenile hormone analog. We found that the three compounds impaired oogenesis and molting and demonstrated that MET also interfered with embryogenesis (Gauthier et al., unpublished data). The evolutionary conservation of RETs across phyla adds to the interest in the RET system in *G. fossarum* and a better understanding will help to identify new targets for EDC in crustaceans.

In this study, aiming to better understand the RET metabolism of *G. fossarum*, our first goal was to identify and record the changes in concentrations of several RETs in unexposed females during their reproductive cycle. To this date, few studies measured RETs in invertebrates and even less focused on crustaceans (see reviews by Albalat, 2009; André et al., 2014). Endogenous isomers of RETs such as RA (9-*cis*/all-*trans*), retinaldehyde (RALD, 13-*cis*/all-*trans*), and ROL (13-*cis*/all-*trans*) were found in ovaries, regenerating blastemas as well as in eyestalks in *Litopenaeus vannamei*, *Scylla serrata* and *Uca pugilator* (Liñan-Cabello et al., 2003, 2004; Hopkins et al., 2008; Venkaiah et al., 2019). Retinoids have never been investigated in *G. fossarum*, let alone suggested as biomarkers in crustaceans. In this study, we perfected a method to analyze the RETs based on one we used on honey bees (Gauthier et al., 2018) and developed a new UHPLC MS/MS analytical method to separate, identify and measure endogenous RETs in unexposed *G. fossarum* during the reproductive cycle of females. The second goal of our study was to evaluate the effects of exposure to atRA and CIT as well as to two pesticides MET and GLY on the RET levels. We, therefore, exposed *G. fossarum* females in controlled conditions to these compounds at concentrations where they were previously reported to elicit perturbations in reproduction and development (Gauthier et al., unpublished data). More precisely, we measured levels of several RETs at three meaningful times during their reproductive cycle: at the beginning of the cycle (day 0), during late secondary vitellogenesis (day 17) and at the end of the reproductive cycle (day 27) which corresponds to the reproductive stages AB, C2 and D2, respectively (Geffard et al., 2010). Our interest in atRA and CIT lies in their ability to influence the concentration of RA. Using MET and GLY will allow us to examine the perturbation of RET levels by pesticides known or suspected to intervene with the RET signaling. This study acquires for the first-time knowledge of the RET metabolism in this specie which is vital for the future development of RET-based biomarkers in crustaceans. In parallel, it will also contribute to the understanding of endocrine control by endogenous RETs and perturbation by xenobiotics in a crustacean sentinel specie.

## 2. Material and methods

### 2.1. Reagents and materials

#### 2.1.1. Exposure solutions

All-*trans* RA ( $\geq 98\%$ ), CIT ( $\geq 98.0\%$ ), GLY ( $\geq 98.0\%$  n-(phosphomethyl)glycine, PESTANAL®), and MET ( $\geq 99.0\%$  PESTANAL®) were purchased from Sigma Aldrich (Saint Quentin Fallavier, France). All-*trans* RA, CIT, and MET were dissolved in acetone, and GLY was dissolved in groundwater to constitute the working solutions. Working solutions for all four compounds were made the day before the start of the experiments, kept in a  $-80^\circ\text{C}$  freezer, and used daily to expose gammarids. To acquire information on the degradation of compounds in exposure media, we analyzed the concentrations of each compound sample ( $n_{\text{total}} = 3$ ) by UHPLC-MS/MS after 0, 4, 8, and 24 h (Fig. S1). All-*trans* RA was analyzed by injecting water samples directly into the UHPLC-MS/MS system (Table S2 and S3) while MET was subject to a liquid-liquid extraction before injection (Note S1 and Table S4). Glyphosate analyses were performed by La Drôme Laboratoire (Drôme, France) using FMOC-Cl derivatization before mass spectrometry quantification. For CIT, experimental challenges (limited reproductibility and ionization for CIT in aqueous media) led to the quantification of CIT in the acetone stock solution which was used to make the exposure media. Therefore, and for CIT only, we quantified the concentration of the stock solution in acetone (later diluted in forage water to constitute the exposure media) based on the sum of both stereoisomers (neral and geranial, Table S1).

Three experimental units (5 L-bottles containing 4 L of aerated forage water) per compound were contaminated to the previously mentioned concentrations and were kept in exposure conditions for 24 h. In the text, we refer to initial concentration at T0 as nominal values:

40 µg/L for RA (confirmed  $39.9 \pm 5.3$  µg/L), 20 µg/L for MET (confirmed  $18.3 \pm 2.8$  µg/L), 5 mg/L for GLY (confirmed  $5.44 \pm 0.34$  µg/L) and 50 µg/L for CIT. The experimental concentration of the CIT stock solution was confirmed to be within 2% of the theoretical concentration. The degradation results motivated our choice of renewing the exposure media manually for all compounds as frequently as possible (every 24 h) for the entirety of the experiment. Analytical standards, solvents, and extraction reagents.

Salts for QuEChERS extraction (EN method, #CAT 5982–0650) were purchased from Agilent technologies (Les Ulis, France). This citrate buffer contains 61.5%<sub>w/w</sub> MgSO<sub>4</sub>, 15.4%<sub>w/w</sub> NaCl, 15.4%<sub>w/w</sub> C<sub>6</sub>H<sub>5</sub>Na<sub>3</sub>O<sub>7</sub> • 2 H<sub>2</sub>O, and 7.7%<sub>w/w</sub> C<sub>6</sub>H<sub>5</sub>NaO<sub>8</sub> • 1.5 H<sub>2</sub>O. Analytical standards for 9-*cis* RA (≥ 99%), 13-*cis* RA (≥ 99.9%), all-*trans* RA (≥ 98%), all-*trans* ROL (≥ 97.5%), all-*trans* RALD (≥ 99%), retinyl acetate (≥ 99.5%), ascorbic acid (99%), butylated hydroxyanisole (BHA, ≥ 98.5%) and hexane were purchased from Sigma Aldrich. Analytical standards for 9-*cis* RALD (≥ 98%), 11-*cis* RALD (≥ 80%), 13-*cis* RALD (≥ 95%), 9-*cis* RA methyl ester (≥ 96%), 13-*cis* RA methyl ester (≥ 95%), 9-*cis* ROL (≥ 95%) and 13-*cis* ROL (≥ 96%) were purchased from Santa Cruz Chemicals Inc (Heidelberg, Germany). Analytical standards for all-*trans* RA methyl ester (≥ 99%), and all-*trans* RA-d5 (major, ≥ 96%) were purchased from Toronto Research Chemicals Canada (Toronto, Canada). Acetone and heptane were purchased from EMD Millipore (Darmstadt, Germany), acetonitrile was from Honeywell, Ridel-de Haën (Seelze, Germany), diethyl ether from Merck KGaA (Darmstadt, Germany), formic acid and isopropanol from Biosolve Chimie (Dieuze, France) and water from Fisher Scientific (Illkirch, France). Only LC-MS grade solvents were used for chromatography.

### 2.1.2. Retinoic acid metabolites synthesis

Retinoic acid metabolites (13-*cis* 4-oxo RA and all-*trans* 4-oxo RA) were synthesized according to McKenzie et al. (1979) with slight modifications. Briefly, a 2 mL solution of n-heptane containing atRA (130 mg/L) and iodine (25 mg/L) was irradiated overnight under a 8 W Light-Emitting Diode yellow bulb (560 – 590 nm), evaporated to dryness the following day, and resuspended in diethyl ether. This solution was further evaporated to dryness, resuspended in acetonitrile, and was assessed by HPLC-UV (Table S5) to ensure the presence of the two RA metabolite peaks.

## 2.2. Gammarid collection and acclimatization

Gammarids used in this study were collected in August and September 2020 in a control site, waterbed cress (Ain, France) known for its very low anthropogenic pressure. Gammarids were collected and acclimatized. Briefly, individuals selected by sieving (2–2.5 mm) were brought back to the laboratory for a 10-day acclimation period (aerated 30 L aquariums, 16 °C ± 0.5 °C, 16/8 h light/dark cycles), fed ad libitum with *Alnus* spp. leaves and provided with continuous fresh and aerated groundwater. Groundwater was sourced from a forage well connected to the water table in Villeurbanne (Rhône, France), filtered through sand and a 10 µm filter with activated charcoal.

## 2.3. Gammarid exposures

Two consecutive sets of exposures were conducted in 5 L bottles containing 4 L of groundwater as well as a square mesh (5 × 10 cm) for enhancement and to allow dispersion of gammarids. During the experiments, groundwater was changed daily, gammarids were fed ad libitum with *Alnus* spp. leaves and temperature was maintained at 16.1 °C ± 0.26 °C. To ensure that *G. fossarum* females used in our study were all synchronized in their reproductive and molting cycles, we selected females in AB reproductive stage (Geffard et al., 2010) from a large number of couples (± 1600 per exposure). After 48 h, all freshly molted females that had laid their eggs in their marsupium were sampled and then distributed randomly in the 5 L bottles (± 125 individuals per

bottle). On day 0, before allocating gammarids to each condition, samples (n = 3) were taken from the gammarid pool to measure baseline RET levels at the beginning of the reproductive cycle. On day 17 (stage C2) and day 27 (stage D2) which respectively corresponds to the late second vitellogenesis and the end of the reproductive cycle, surviving gammarids from one 5 L-bottle per condition were counted, rinsed, and shared into three samples (n = 3). Two sets of experiments were conducted. For the first set of experiments, six 5 L-bottles were separated into three conditions solvent group (SOLV), atRA (50 µg/L), or MET (20 µg/L). For the second set of experiments, four 5 L bottles were allocated to SOLV and CIT (50 µg/L) and two were allocated to CTRL and GLY (5 mg/L). We used acetone which is the solvent routinely used in our lab for similar experiences because it was previously demonstrated as having no effects on reproductive parameters in *G. fossarum* (Gouveia et al., 2018). In graphs, SOLV groups are labeled as "unexposed". Because of limited access to biological material, gammarids from CTRL and GLY conditions were collected on day 27 only, whereas other conditions were sampled on day 17 and at 27. Survival data are reported in Table S6. Upon collection (day 0, 17, or 27), all samples were weighted and frozen at – 80 °C until RET analysis.

## 2.4. Retinoid extraction and quantification

### 2.4.1. Hybrid QuEChERS extraction procedure

Samples (± 0.4 g of tissues, approximately 30 individuals) were homogenized on ice with a POLYTRON® Immersion Disperser for 20 s in 2 mL of phosphate buffer solution (PBS, 0.5% ascorbic acid, pH 7.5). After adding 100 µL of a solution of internal standard (2 mg/L, all-*trans* RA-d5), the tube was vortexed for 5 s and extracted twice with organic solvents; 2 mL of ACN (0.1% BHA) and hexane (0.1% BHA) were added in combination with 1.3 g of QuEChERS salts. The tube was vortexed 30 s and centrifuged for 2 min (3000 × g, 4 °C), and all the supernatant was collected. The second extraction was carried out by adding 2 mL of ACN (0.1% BHA) and 2 mL of hexane (0.1% BHA) without salts. Again, the tube was vortexed and centrifuged for 2 min (3000 × g, 4 °C), and the supernatant was pooled with the previous one. The combined supernatants were centrifuged for 2 min (4000 × g, 4 °C). Then, 3 mL of hexane (upper phase) and 3.5 mL of ACN (lower phase) were evaporated to dryness at 50 °C under constant nitrogen flow and resuspended in 450 µL of isopropanol. The extract was centrifuged for 10 min (10 000 × g, 4 °C), and the supernatant was transferred to a 1.5 mL microtube and aliquoted to 4 amber vials with inserts (100 µL each). All the steps described in this procedure were carried out in dark amber vials under yellow or dim light.

### 2.4.2. Analysis by liquid chromatography-tandem mass spectrometry

Liquid chromatography was performed using an Acquity UHPLC H-Class (Waters, Saint-Quentin en Yvelines, France). The separation was achieved with a Zorbax eclipse plus PAH column 2,1 × 100 mm, 1,8 µm from Agilent Technologie (Avondale, USA) and a Krudkatcher prefilter from Phenomenex (Le Pecq, France) in gradient mode using H<sub>2</sub>O (0.1% formic acid) and ACN (0.1% formic acid) as phase A and B, respectively. The chromatographic system was coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters) and electrospray ionization (ESI) was performed in the positive mode. Additional parameters relative to the chromatography and ionization are listed in Table S7 and S8. For all analytes, spectrometric investigations were carried out in multiple reaction mode with the molecular ion peak as the parent ion. However, parent ion for retinol (MW: 286.3 g/mol) and retinyl acetate (MW: 328.5 g/mol) were *m/z* = 269.3 and 269.0 following a loss of H<sub>2</sub>O and lateral COOCH<sub>3</sub> chain, respectively. IntelliStart™ was used to automatically determine the 2 main transitions with optimal values of cone voltage (V) and collision energy (eV). The first and second transitions were used for quantification and confirmation, respectively. Identification of a compound was confirmed when the two main transitions were detected with a signal/noise ratio over 3 (S/N > 3), and that retention

time (RTs), and ion ratios were within a 2%, and 30% variation compared to their analytical standard, respectively (Table S9 and Fig. S2). Limits of quantification (LOQ) and detection (LOD) were estimated as the concentration for which a signal-to-noise ratio of 10 for the first transition and 3 for the second transition was obtained, respectively (Table S10). In samples where the signals were found under the LOQ and over the LOD, we assigned a concentration halfway between these two values. In cases where the first transition was present ( $S/N > 3$ ), but no second transition was found, we still assigned a value equivalent to the LOD/2.

#### 2.4.3. Quantification

We determined the concentrations of RETs in the extracts using a standard addition method following a 4-point calibration curve for each sample. The four points were constituted of 100  $\mu\text{L}$  of the extract, and 25  $\mu\text{L}$  of ACN for the first point injected (P1), whereas the following 3 points (P2, P3, and P4) were constituted by adding 25  $\mu\text{L}$  of a RET mix containing increasing concentrations of RA metabolites (all-trans 4-oxo RA, 13-cis 4-oxo RA), all-trans ROL, 11-cis RALD, all-trans RALD, and 13-cis RALD. The 3 points in the calibration curves for each RET were calculated so the signal for P2, P3, and P4 would vary between 2 and 15 times the signal of P1. The linearity was assessed and the slope and the y-intercept were used to estimate the concentration of RETs in P1. Retinoid concentrations are expressed as ng/g of tissues except for metabolites (13-cis 4-oxo RA and all-trans 4-oxo RA) that were synthesized together and for which the exact oxidation and photoisomerization yields were not determined experimentally. For this reason, values are given as AUC/g of tissue. In this study, mean concentrations are presented along with their respective standard error of the mean (SEM) to inform on the value of tissular concentrations themselves.

#### 2.5. Statistical analyses

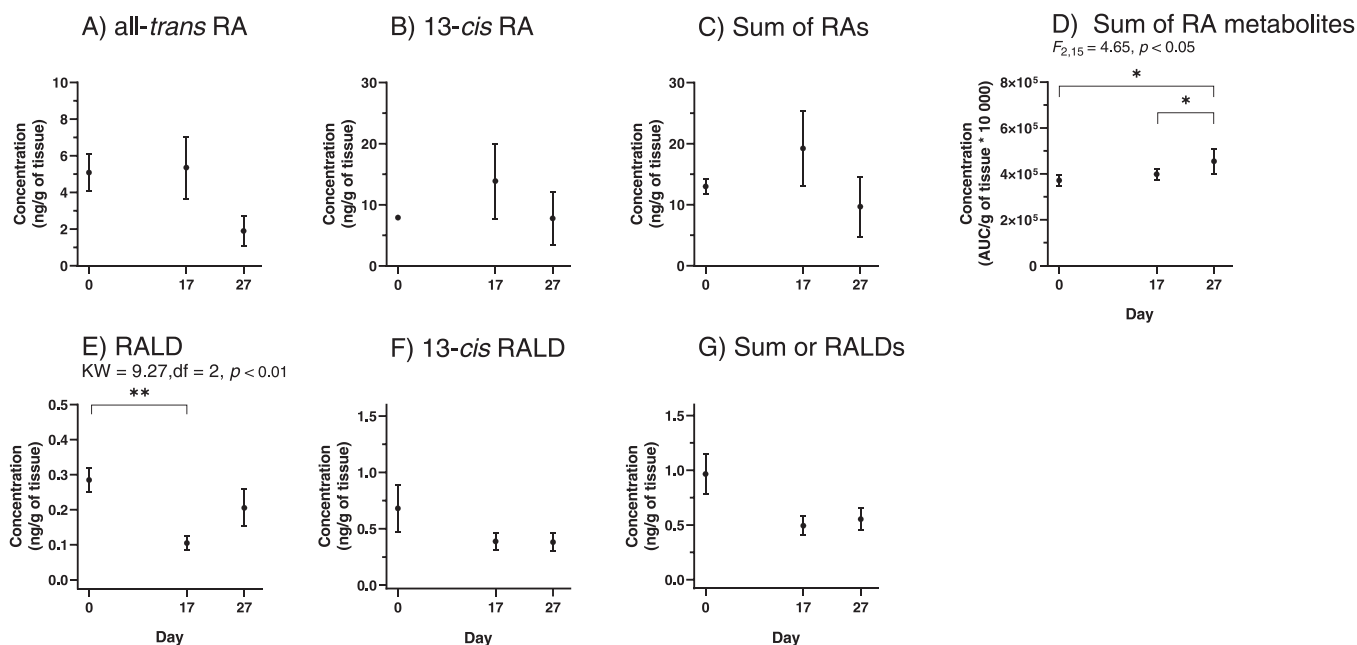
Concentrations of RETs (day 0, 17, and 27) and survival (day 17 and 27) were compared between the first and second set of experiments using the Wilcoxon-Mann-Whitney non-parametric test and Fisher exact test ( $2 \times 2$  contingency table), respectively. Given the absence of significant results ( $p < 0.05$ ) for mortality and baseline RET levels between the unexposed groups for the two sets of experiments, data from both

experiments were pooled. Among unexposed females, concentrations of RET (all-trans/13-cis RA, sum of RA isomers, sum of RA metabolites, all-trans/13-cis RALD, and sum of RALD isomers) were tested for normality of distribution and homoscedasticity with Shapiro-Wilk and Levene tests, respectively. When data complied with normality and homoscedasticity, they were tested using the GLM (General Linear Model) one-way analyses of variance (ANOVA). Significant models ( $p < 0.05$ ) were followed by multiple pairwise "t" tests between day 0, day 17, and day 27. We used non-parametric Wilcoxon-Mann-Whitney tests to evaluate the effects of atRA, CIT, MET, and GLY on RETs by comparing data between each exposed group with their respective control group and for day 17 and day 27. In addition, survival and proportions of RA isomers (all-trans/13-cis RA), and RALD isomers (all-trans/13-cis RALD) with RA metabolites between unexposed and exposed groups were compared for each endpoint (days 17 and 27) with the Fisher exact test ( $2 \times 2$  contingency table). The  $p$ -values were considered significant if they were lower than 0.05 and were considered marginally significant between 0.05 and 0.1. All statistical analyses were performed with RStudio (version 1.4.1717).

### 3. Results and discussion

#### 3.1. Changes in endogenous retinoids throughout the reproductive cycle

To establish RET baseline levels in *G. fossarum* females, several endogenous RETs were investigated and some were identified throughout the reproductive cycle (Fig. 1, Table S10). To begin, the internal standard (at-RA-d5) signal variation measured in all samples ( $\pm 20.3\%$ ) confirmed the robustness of the extraction and injection procedure. Most RETs we identified are consistent with those observed in crustaceans and summarized by André et al. (2014). However, we identified for the first time in crustacean tissues four RETs: 13-cis RA, 11-cis RALD, and the metabolites all-trans 4-oxo RA and 13-cis 4-oxo RA. In addition to those, we found in *G. fossarum* tissues isomers of RA (all-trans and 13-cis) and RALD (all-trans and 13-cis). Retinoic acid metabolites (13-cis 4-oxo RA and all-trans 4-oxo RA) and RALD isomers (all-trans, 11-cis, and 13-cis) were quantified in 80–100% of samples throughout the reproductive cycle and values for RA isomers (all-trans and 13-cis) were obtained for 86–91% of unexposed females (Table S10).



**Fig. 1.** Concentrations (means  $\pm$  SEM) of retinoid species in unexposed *G. fossarum* females. Each chemical analysis was conducted on approximately 30 individuals ( $n = 6$ ). \*  $p < 0.05$ , \*\*  $p < 0.01$ .

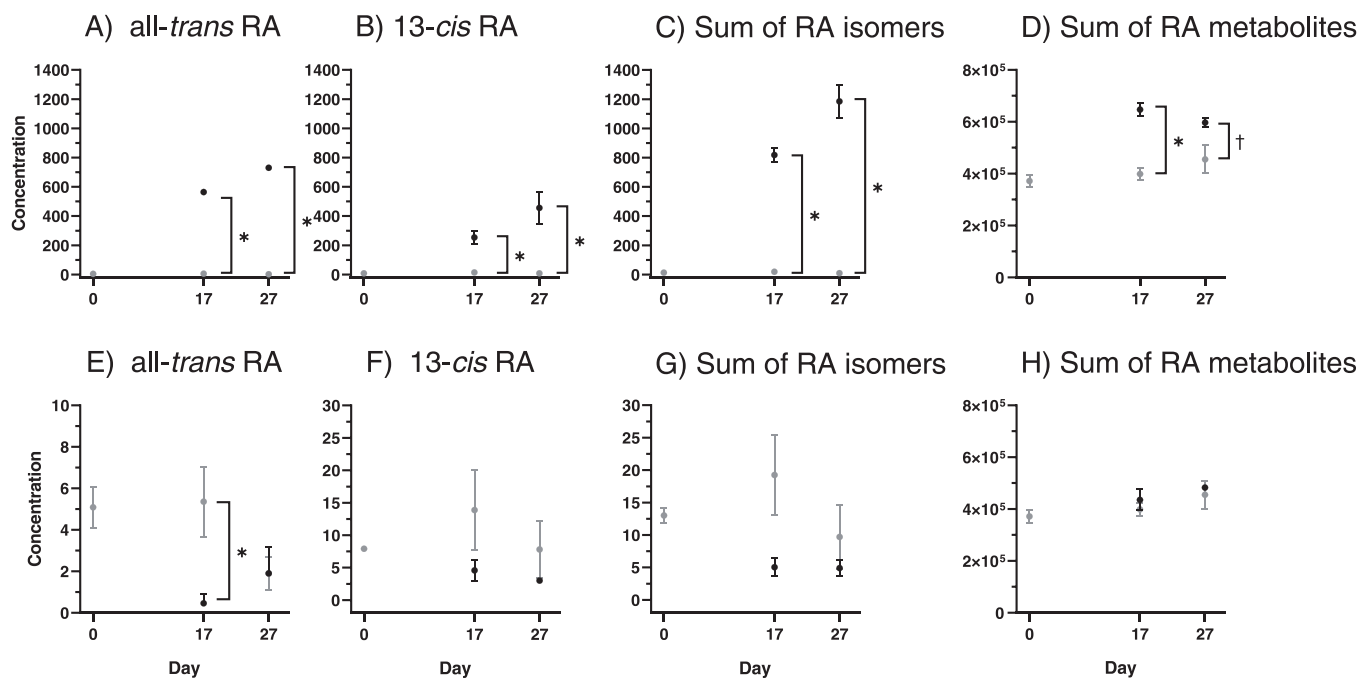
It is noteworthy to mention that in all chromatograms, the major RA metabolite found was the 13-*cis* 4-oxo RA isomer. Also, although our method allowed for the identification and measurements of 9-*cis* RA, ROL isomers (all-*trans*, 9-*cis*, and 13-*cis*), 9-*cis* RALD, RA methyl esters isomers (all-*trans*, 9-*cis*, and 13-*cis*), and retinyl acetate standards, no signals were found for any of these compounds in *G. fossarum* tissues. Therefore, we assume that they were either absent in *G. fossarum* females or found in concentrations under their respective LOD (Table S10). Consequently, we presume that RA comes from RALD obtained directly from precursor CARs. Retinoids were only measured in crustacean tissues by a few authors (Liñan-Cabello et al., 2003, 2004; Hopkins et al., 2008). Liñan-Cabello et al. (2003) and Liñan-Cabello and Paniagua-Michel (2004) reported *L. vannamei* ovarian concentrations of all-*trans* RALD and 13-*cis* RALD ranging from 100 to 180 ng/g and from 820 to 1000 ng/g of tissue, respectively. They also reported concentrations of all-*trans* RALD in eyestalks (approximately 300 ng/g) and the hepatopancreas (from 160 ng/g to 300 ng/g) and 13-*cis* RALD in eyestalks at concentrations similar to all-*trans* RALD (300 ng/g). In RET analysis conducted on whole organism's homogenates of honey bee *Apis mellifera*, we reported concentrations of RALD (sum of 9-*cis*, 13-*cis*, and all-*trans*) ranging from 174.6 to 236.3 ng/g of tissue (Jumarie et al., 2017; Gauthier et al., 2018; Mahé et al., 2021). In these studies conducted on crustaceans and insects, RALD isomers were found at much higher concentrations than those reported in our study in unexposed *G. fossarum* females (all-*trans* RALD: 0.10–0.29 ng/g, 13-*cis* RALD: 0.38–0.69 ng/g). Given the smaller masses of gammarids compared to shrimps, our analyses were conducted on whole organism homogenates ( $\pm 30$  individuals per sample) which may partially explain the discrepancy between concentrations found in accumulating organs and gammarids whole-tissue homogenates. Although RALD levels were lower in the gammarid compared to bees, concentrations of RA isomers ( $\pm 7$ –21 ng/g of tissue) are closer to those of *A. mellifera* ( $\pm 39.3$ –71.9 ng/g of tissue) but showed high SEMs. Retinoic acid is essential in the oogenesis of many animals and is conserved across phyla (Wallacides et al., 2009; Adolphi et al., 2016; Li et al., 2016; Peng et al., 2020). It is also essential in the oogenesis of *G. fossarum* females which bear a variable number of oocytes (10–26) and embryos (10–18) (Gauthier et al., unpublished data). It is, therefore, reasonable to assume that the discrepancy in oocyte and embryo bearing influences the physiological need for a specific RA isomer which could partially explain why RA, but not other RETs, had larger SEMs.

Overall, concentrations of RA metabolites raised throughout the cycle while RALD levels decreased in the first half of the cycle. More specifically, we discuss the events in two distinct phases during the reproductive cycle. The first phase (days 0–17), during which most of the vitellogenesis occurs in *G. fossarum* (between days 0 and 17), is characterized by steady atRA levels with a slight, but non-significant increase in 13-*cis* RA levels (Fig. 1A–C). During this phase, while RA metabolites showed a 7% significant increase (Fig. 1D), a 62% diminution of RALD isomers occurred (Fig. 1E). In the second phase (days 18–27), when most of the vitellogenesis ceased and before molting, the levels of RALD (all-*trans* and 11-*cis* isomers) raised back to the initial levels (Fig. 1E), but the sum of RALD isomers showed no fluctuation (Fig. 1F–G). During this same period, RA metabolites increase significantly by 22% (Fig. 1D) possibly as a result of the non-significant yet important diminution of approximately 50% of all measured isomers of RA (Fig. 1A–C). The need for specific RA isomers may expectedly vary during the reproductive cycle of *G. fossarum* females to sustain various specific physiological processes. The observed reduced production of RA oxidated metabolites before day 17 may be a manifestation of a lower RA catabolism while the marked increase of these metabolites between day 18 and 27 could represent a higher RA catabolism. This could plausibly result in an increased RA concentration in the first phase and a decrease in the second phase. The expression of RXR also displays a similar trend in expression profile variations throughout the reproductive cycle of *G. fossarum* females (Gouveia et al., 2018). Gouveia et al.

(2018) showed that RXR expression is heightened at the beginning of the cycle (AB) and gradually lowers until the C1 stage which is reached between days 10–15 at 16 °C (Chaumot et al., 2020). For vertebrates, a complex crosstalk network relying on the differential affinities of RA isomers with isoforms of RXR and RAR is responsible for the trans-activation of specific signaling pathways which sustains crucial physiological functions and therefore constitute a way to orchestrate and regulate these functions. Therefore, the balance between RA isomers may be of primary importance as RXR binds RA isomers with variable affinity. For this reason, and more largely to gain insight into the metabolic conversion between each form, we also investigated ratios of several RETs. In unexposed females, ratios between all-*trans* and 13-*cis* RA isomers were maintained at each endpoint of the reproductive cycle notably 28:72 on day 17 and 20:80 on day 27 (data not presented). Surprisingly, in our study, we measured very low levels of RALD isomers ( $\pm 0.1$ –0.3 ng/g) compared to their oxidated products, RA isomers ( $\pm 9.7$ –19.2 ng/g). According to our estimations, between days 0 and 17, the mean tissular concentrations of RA isomers increased from 13.0 to 19.2 ng/g. To sustain such an augmentation of RA levels and given the low concentrations of the immediate RA precursors (e.g. RALD isomers) reported in our study, one may suppose that large stocks of RALD precursors (e.g. CARs) would be required between days 0 and 17. In crustaceans, CARs are accumulated in the ovaries along with the vitellogenin, a lipoglycocarotenoprotein, and are further incorporated in the oocyte (see review by Liñan-Cabello et al., 2002). As stated before, in *G. fossarum*, most of the primary and secondary vitellogenesis is completed upon day 17 (Geffard et al., 2010). Hence, we believe that between days 0 and 17, while CARs are mobilized in ovaries for the later incorporations in oocytes, they must also sustain the physiological requirements in RA (e.g. for oogonial differentiation), and consequently, would explain the significant decrease in RALD observed during this time frame. Given the high reactivity of the aldehyde group with cellular protein thiols and amines (O'Brien et al., 2005), it is also biochemically reasonable to assume that RALD is kept in low concentrations to avoid unwanted and excessive reactions with intracellular proteins. In healthy *G. pulex* and *G. roeseli* females, the total CAR contents were estimated to be  $203.1 \pm 130.2$  and  $188.7 \pm 71.3$   $\mu\text{g/g}$  of tissue, respectively, and consist of approximately 25% of esterified  $\beta$ -carotene (Gaillard et al., 2004). That being said, if tissular concentration of this provitamin A  $\beta$ -carotene esters was to be in a similar range in *G. fossarum*, it would most likely suffice the 6 ng/g increase in RA isomers between day 0 and 17. However, CAR analysis in *G. fossarum* females would be required to support this hypothesis. Another explanation could be that different forms of RETs (e.g. dehydroretinal), possibly leading to the formation of RA, have not been identified in our study (La Frano et al., 2017).

### 3.2. Effects of retinoic acid and citral on retinoid profiles

Having previously reported reproductive and developmental defects in oogenesis and molting in female *G. fossarum* exposed to atRA and CIT, these compounds were further examined for their capacity to simulate excess or deficiency in RA, respectively, and to attest to the mediation of RETs in these disorders. Exposure to atRA caused a significant increase in tissular concentrations of both RA isomers (all-*trans* and 13-*cis*) on day 17 and day 27 (Fig. 2A–C). In conjunction with the increased RA tissular concentrations, increased RA metabolite levels were also observed on day 17 and day 27. However, on day 27, the increase was only marginally significant. Although unsurprising given the known RA catabolism in vertebrates, this is coherent with the increased RA concentrations and suggests that the catabolism of all-*trans* and 13-*cis* RA in gammarids also involves the formation of all-*trans* 4-oxo and 13-*cis* 4-oxo RA. Following exposure to CIT, a known RA synthesis inhibitor, the obtained results were quite different and are supported by the complete inhibition of RALD conversion to RA already observed by Hopkins (2001) in *Uca pugnator* limb buds. On day 17, CIT significantly reduced the concentration of atRA and had no significant effects on

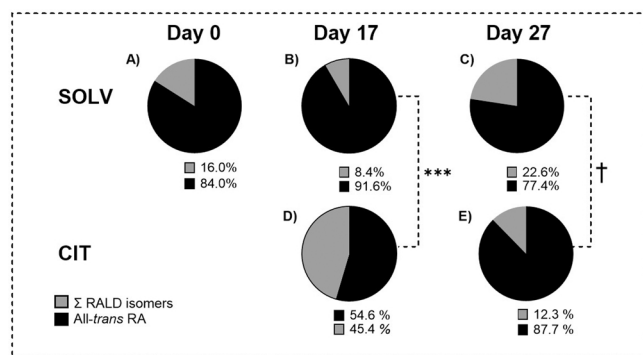


**Fig. 2.** Concentrations (means  $\pm$  SEM) of retinoic acid isomers (ng/g of tissue) and metabolites (AUC/g of tissue) in *G. fossarum* females exposed to all-*trans* RA (A-D) and CIT (E-H). Each chemical analysis was conducted on approximately 30 individuals ( $n = 3$ , except for day 0 where  $n = 6$ ). Values and lines are presented in grey for unexposed and in black for exposed females.  $\dagger p < 0.1$ ,  $* p < 0.05$ .

13-*cis* RA levels (Fig. 2E-G). However, the sum of RA isomers was statistically lower on day 17 ( $p < 0.05$ ) and on day 27 ( $p < 0.05$ ) when compared to day 0 (not shown on the graph, KW = 8.7, df = 2,  $p < 0.05$ ). This was not the case for unexposed gammarids. In addition, the estimated 75% diminution in the mean sum of RA isomers following exposure to CIT on day 17, although not statistically significant, is massive. As expected, the ratio between the sum of RALDs and at-RA in *G. fossarum* was highly significant on day 17 which shows a drastic change in the proportions of these species following exposure to CIT (Fig. 3). This was not the case for the proportions of both isomers of RALD and 13-*cis* RA. Overall, ratios of at-RA and isomers of RALD in CIT-exposed females clearly indicated a more prominent presence of RALD as well as a lower proportion of at-RA which supports the inhibitory action of CIT on a putative *G. fossarum* RALDH. Finally, following CIT exposure, concentrations of RA metabolites in female gammarids stayed in the same range as the unexposed ones for the entirety of the cycle. Taken together with the previously observations by Gauthier et al. (unpublished data), we demonstrated that at-RA and CIT are good candidates to simulate an excess and a deficiency in RA, respectively, for

*G. fossarum*. In turn, the excessive concentrations of RA isomers throughout the reproductive cycle hindered conversion from RALD to RA resulting in insufficient tissular concentrations of at-RA specifically during the first half of the cycle resulting in oogenesis and molting impairments.

Despite the contrasting effects of at-RA and CIT on RA isomers and metabolites, they both affected negatively the concentrations of RALD isomers on day 27, except for CIT which did not reduce the concentration of 13-*cis* RALD (Fig. S3). In comparison with the unexposed groups, the diminutions of RALD that occurred naturally throughout the cycle showed a tendency to be more pronounced at the end of the reproductive cycle. Although at-RA and CIT affected similarly the concentrations of RALD at 27, these effects occurred in different contexts. In at-RA exposed gammarids, the concentrations of RALD are reduced while concentrations of RA and RA metabolites increase, attesting to an increased RALD oxidation to RA and RA metabolites. However, in CIT-exposed gammarids, the RALD concentrations do not reflect the increase in RA and RA metabolites levels suggesting that RALD production may be hindered upstream of RA formation. As of today, it is challenging to speculate on the possible cause of RALD diminutions following exposure to at-RA and CIT. In vertebrates, the enzyme BCMO is responsible for the oxidative cleavage of pro-vitamin A CARs to RALD isomers, but we have little knowledge of the regulation by BCMO in crustaceans. Foliar treatment with CIT has also been shown to repel the aphid *Myzus persicae* and to reduce the probing activity from CIT-treated areas (Dancewicz et al., 2020). Citral-induced feeding disturbances could reduce the CAR intake and eventually lead to CAR depletion and reduced RALD levels. However, as stated before, besides a plausible CAR deficiency at the beginning of the exposure, CARs are stored in high concentration and such depletion would surprisingly arise after only 27 days. Again, chemical analyses would be necessary to investigate the CAR status of exposed gammarids and to gain better insight into these RALD precursors.



**Fig. 3.** Proportions (%) of the sum of RALD isomers to all-*trans* retinoic acid in *G. fossarum* females exposed to citral. Percentages were calculated with the mean concentration of each compound. Each chemical analysis was conducted on approximately 30 individuals.  $\dagger p < 0.1$ ,  $*** p < 0.001$ .

### 3.3. Effects of MET on retinoid profiles

In our study, the tissular concentrations of RETs females did not

differ significantly between MET-exposed and unexposed females, but changes in ratios between RETs species were observed (Figs. 4 and 5). Although not statistically different, MET caused a 55% reduction in the sum of RA isomers at day 17 (Fig. 4A-C), and reductions of approximately 45% in 13-*cis* RALD concentrations throughout the reproductive cycle (Fig. 5A-C). On day 17, MET showed marginally significant disruption of the proportions of RA isomers and RA metabolites (Fig. 4). In this case, concentrations of RA isomers were disproportionately smaller when compared to the unexposed group. Furthermore, on day 27, MET impaired the balance between the two RA isomers (all-*trans* and 13-*cis* RA, Fig. 4G-I), as well as the ratio between the sum of RALD isomers and atRA (Fig. 5F-H). Following exposure to commercial formulations of atrazine and thiamethoxam in PLHC-1 and ZFL hepatic fish cell lines, similar disorders in RA isomerization resulted in increased biologically active RA isomers (atRA and 9-*cis* RA) were also reported (Hanna et al., 2022). Altogether, the reported ratios in our study indicate an over-representation of atRA compared to 13-*cis* RA, and a higher proportion of atRA compared to the sum of RALD isomers. This could be the result of 1) a disorder in the RA isomerization in favor of the all-*trans* isomer, 2) a perturbed RA catabolism, or 3) increased oxidation of RALD to atRA. It is noteworthy to highlight that the all-*trans* isomer of RA,

measured in higher proportions in our study, may act as a much more affine isomer for RXR compared to the 13-*cis* isomer (Allenby et al., 1993). Further studies focusing on RA isomerization and catabolism as well as the bioconversion from RALD to RA in MET-exposed *G. fossarum* would be required to understand the source of such perturbation. In crustaceans, there is evidence for the implication of RXR in RET-induced effects on ecdysteroidogenesis, methyl farnesoate synthesis, ovarian maturation, and limb regeneration (Hopkins et al., 2008; Girish et al., 2018). Hence, the identification, for the first time, of a 13-*cis* RA isomer in crustacean tissues highlights a possibly distinct regulation of RXR-mediated pathway by isomerization of RA in *G. fossarum* and more largely in crustaceans. In the crustacean *Uca pugnator*, depending on the isoform of RXR it is heterodimerized with, 9-*cis* RA may influence positively or negatively the binding of the ecdysone receptor (EcR) to the radiolabeled metabolite of 25-deoxyecdysone ponasterone A (Hopkins et al., 2008). The isomerization of RA, heterodimerization, and colocalization of RXR isoforms with EcR in targets and secretory tissues may therefore be a way to regulate the ecdysone signaling pathway and have important effects on the reproductive physiology of crustaceans. Besides its effects on the isomerization processes, MET could also act directly on RXR either through direct binding or by affecting its

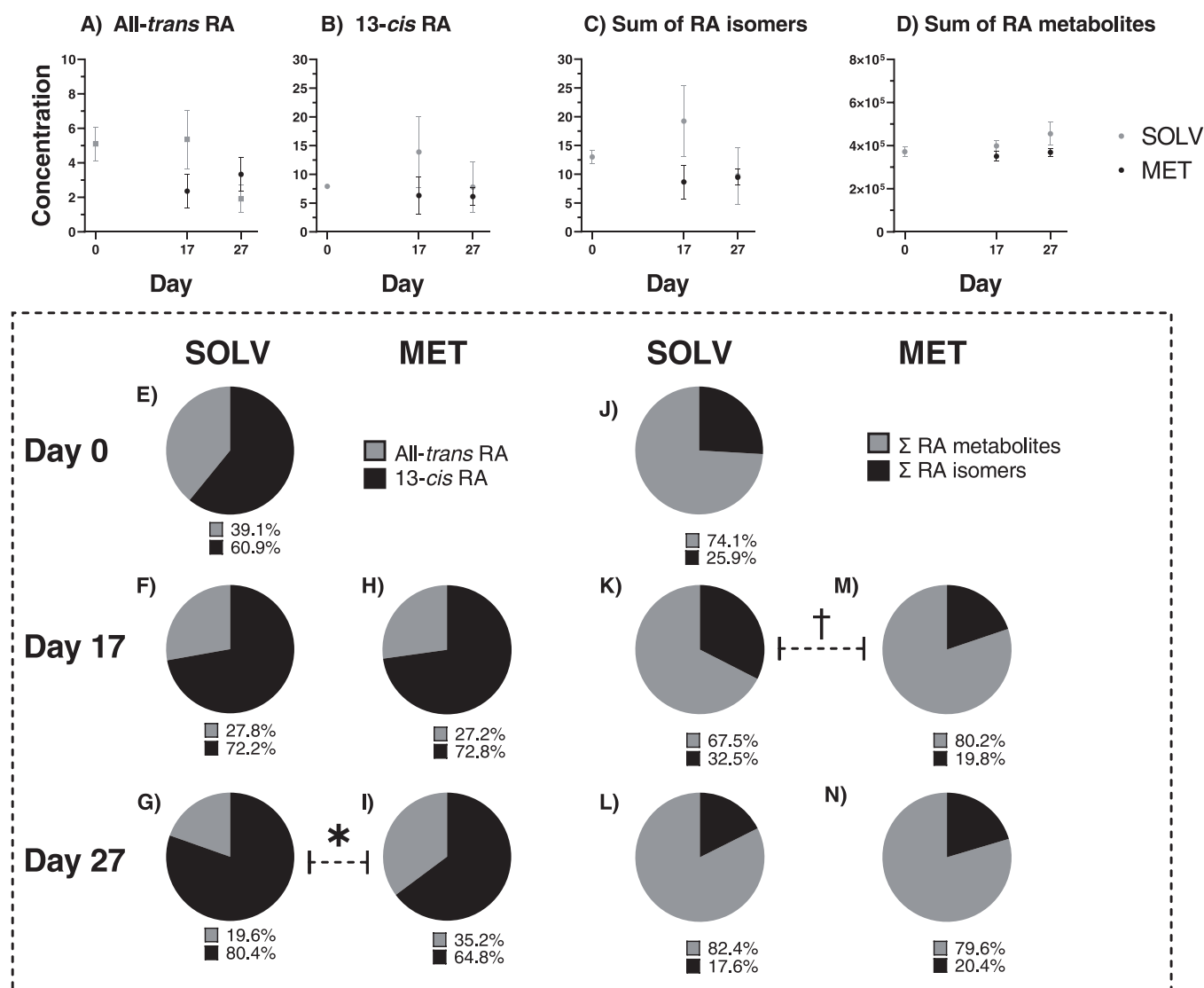
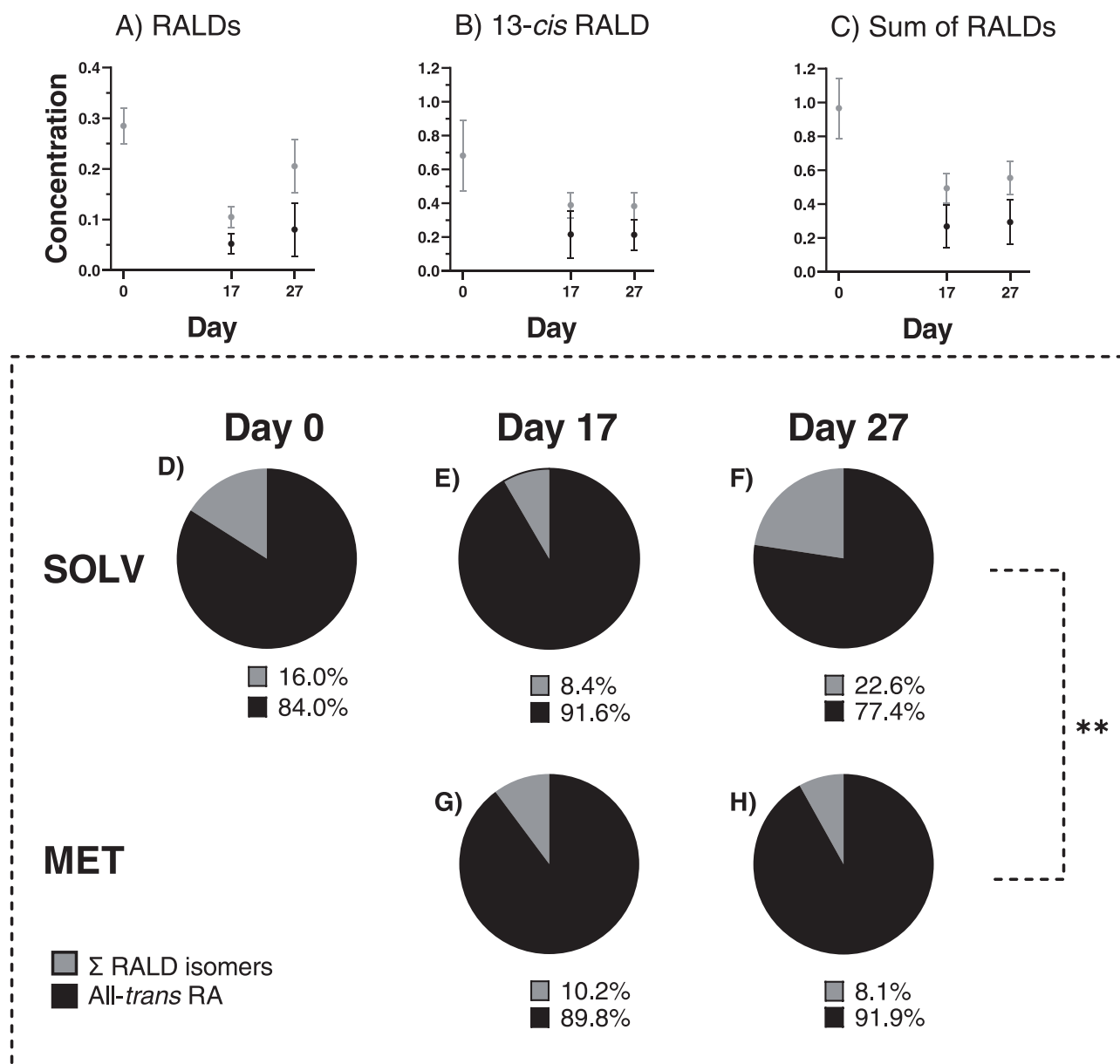


Fig. 4. Concentrations (means  $\pm$  SEM) of retinoic acid isomers (A-C, ng/g of tissue) and metabolites (D, AUC/g of tissue) and their proportions (E-N) during reproduction of *G. fossarum* females exposed to methoprene. Values and lines in A-D are presented in grey for unexposed and in black for exposed females. Each chemical analysis was conducted on approximately 30 individuals ( $n = 3$ , except for day 0 where  $n = 6$ ). †  $p < 0.1$ , \*  $p < 0.05$ .





**Fig. 5.** Concentrations (means  $\pm$  SEM) of retinaldehydes (A-C, ng/g of tissue) and proportions of its isomers with retinoic acid isomers (D-H) throughout the reproductive cycle in *G. fossarum* females exposed to methoprene. Values and lines in A-C are presented in grey for unexposed and in black for exposed females. Each chemical analysis was conducted on approximately 30 individuals ( $n = 3$ , except for day 0 where  $n = 6$ ). \* \*  $p < 0.01$ .

expression. According to a few authors, EDC such as tributyltin could bind to the crustacean RXR and influence the events downstream of its signaling pathway resulting in physiological disorders like molting (see reviews by Nakanishi, 2008; Lagadic et al., 2017). In parallel, some EDCs like pyriproxyfen or piperonyl butoxide were also shown to influence the expression of RXR following 14 days of exposure (Gouveia et al., 2018). However, as we did not measure the expression of RXR in this study, this mechanism of toxicity remains speculative and further studies on the action of MET will be needed to validate our suspicions.

### 3.4. Effects of GLY on retinoid profiles

Glyphosate concentrations known to induce molting defects in *G. fossarum* were also tested to evaluate their effects on RET levels. Fluctuations and ratios of RETs were identical in unexposed and GLY-exposed groups (Fig. S4). In both groups, the sum of RA isomers showed a tendency to lower on day 27 and RA metabolites significantly

increased between days 0 and 27 (Fig. S4). Retinaldehyde also showed a significantly lower concentration on day 27 (Fig. S4). In other words, the technical formulation of GLY at 5 mg/L did not influence the concentrations of RETs nor their proportions in *G. fossarum* females. Nevertheless, these observations confirmed the robustness of the experimental design and analytical method and allowed us to discriminate the toxicodynamics of MET and GLY, two completely different compounds, on RET profiles. As stated before, Hanna et al. (2022) observed the effects of a commercial formulation of GLY-base herbicide (GBH) on the isomerization profiles of RA, and Hedrei-Helmer et al., (2014) also showed that a GBH tended to lower concentrations of tissular ROL. However, our test was conducted with technical formulations of GLY, and ROL was not found in *G. fossarum* which may explain these discrepancies. The previously observed effects of GLY on molting by Gauthier et al., (unpublished data) would therefore not be mediated by RETs. It is also important to reaffirm that the targeted analyses conducted in our study, although enlightening on some RETs in *G. fossarum* females, only

focused on 15 forms that may not represent the entirety of the endogenous REs. Thus, non-target analyses are the wise next step to gain a global and comprehensive portrait of disturbances of the RET profile in *G. fossarum*.

#### 4. Conclusion

In this study, we identified and monitored the levels of several RETs (RA metabolites, RA, and RALD isomers) during the reproductive cycle of *G. fossarum* females when exposed to atRA, CIT, MET, and GLY. In unexposed females, while RA metabolites increased throughout the cycle, RALD isomers decreased during vitellogenesis and remained stable afterward. Then we confirmed that exposure to atRA increased tissular concentrations of RA isomers and metabolites throughout the cycle and that CIT successfully inhibited the conversion of RALD to RA in *G. fossarum* females more markedly during vitellogenesis. These results were associated with the previously observed oogenesis and molting defects in *G. fossarum* females. Whereas MET caused overrepresentation of all-*trans* RA compared to 13-*cis* RA possibly as a result of increased RALD conversion to atRA on day 27, GLY did not influence the RETs of *G. fossarum* females. Taken together with our previously observed results, we suggest that levels of tissular RA must be regulated throughout the reproductive cycle of *G. fossarum* females to allow successful oogenesis and molting and that disturbances of RA levels or isomerization by xenobiotics interferes with the reproductive and developmental physiology of oogenesis and molting in *G. fossarum* females. The effects observed in our study on females does not foretell effects on male or progeny and such investigations should be conducted. Nevertheless, our results support the regulatory roles of RA in biological functions that are also regulated by the crustacean endocrine system. While both pesticides displayed different toxicodynamics on RETs, RA levels were perturbed following exposure to xenobiotics in this crustacean model which makes RETs in *G. fossarum* promising targets for the development of new biomarkers. Additionally, the concentrations of MET tested in our study were similar to what could be quantified in the environment after recommended MET usage (Hershey et al., 1995). Although exposure levels were confirmed by chemical analyses in the aqueous media, quantification of compounds in gammarid tissues would be valuable to better understand the extent of the exposure. Overall, these results suggest RETs as new molecular targets to investigate the toxicodynamics of reprotoxic compounds as well as compounds that affect the developmental physiology and endocrinology in crustaceans. This revealed the interest in RET-based biomarkers in crustaceans to better understand the effects of environmental contaminants on non-target aquatic organisms.

#### CRedit authorship contribution statement

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#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data Availability

Data will be made available on request.

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#### Author contributions

M.G. codesigned the experiments, carried out the laboratory work and statistical analyses, interpreted the results, built the corresponding tables and figures, and wrote the manuscript. G.D., B.G., and F.L. helped with the analytical method developments and analyzed the exposure media. E.V. Supervised the chemical analyses. O.G. codesigned the experiments supervised the project and revised the manuscript. All authors revised and approved the final version of the manuscript.

#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.114602.

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