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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

En Biologie Santé

École doctorale n°168 CBS2 - Sciences chimiques et biologiques pour la santé

Unité de recherche IBMM - Institut des Biomolécules Max Mousseron

Caractérisation des mécanismes moléculaires de la
reprotoxicité radioinduite chez *Caenorhabditis elegans*

Investigating molecular determinants of radioinduced
reprotoxicity in *Caenorhabditis elegans*

Présentée par Elizabeth DUFOURCQ-SEKATCHEFF
Le 07 avril 2022

Sous la direction de Simon GALAS et Sandrine FRELON

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Et maintenant, bonne lecture!

“You have evolved from worm to man, but much within you is still worm.”

Nietzsche

RESUME

Dans l'environnement, les êtres vivants sont chroniquement exposés à diverses sources de rayonnements ionisants, qu'elles soient environnementales ou anthropiques en raison de l'utilisation de l'énergie nucléaire, qui peuvent induire plusieurs modifications dans le fonctionnement des organismes.

Historiquement, l'évaluation du risque radiologique sur les espèces sauvages était incluse dans les études sur la radioprotection de l'Homme, postulant que l'être humain étant l'espèce la plus sensible, la protéger reviendrait à protéger l'ensemble des espèces. Cependant, la problématique autour de la perte de la biodiversité et l'importance de protéger l'environnement en tant que tel a pris de l'ampleur, notamment au travers de la convention sur la biodiversité et la déclaration de Rio au cours de la Conférence des Nations unies sur l'environnement et le développement en 1992 (UN, 1992b, UN, 1992a). Dès lors, de nouveaux critères ont été établis et continuent d'évoluer afin d'assurer une meilleure évaluation du risque chez les espèces non-humaines.

Dans le cadre de l'évaluation des risques radiologiques dans l'environnement, un élément essentiel à la protection des espèces est de pouvoir déterminer des seuils de toxicités (i.e., valeurs de référence) en deçà desquels aucun effet n'est observé chez les espèces. Cependant, la définition de ces seuils est extrêmement variable selon les critères pris en compte. D'une part, la radiosensibilité des espèces s'étend sur cinq ordres de grandeur, ce qui rend complexe la détermination d'un seuil unique de protection pour un écosystème donné. Pour pallier ce problème, on considère alors l'espèce la plus sensible comme référence. Cependant, cette sensibilité varie aussi en fonction de paramètres extrinsèques à l'organisme (facteurs environnementaux), ce qui rend complexe l'extrapolation du laboratoire au terrain. D'autre part, la détermination de ces seuils s'établit principalement sur des effets à l'échelle de l'individu entraînant des conséquences à l'échelle des populations (i.e., mortalité, reproduction, croissance). Or, il a été observé que les effets à l'échelle sub-individuelle (parfois non moins délétère au regard de l'impact sur les populations) surviennent à des seuils beaucoup plus bas que les effets à l'échelle individuelle. De fait, une approche moléculaire de la caractérisation des effets permettrait de mettre en évidence des marqueurs de l'irradiation

plus précoces et sensibles. Enfin, à ces défis complexes vient s'ajouter le problème de manque de données en situation d'exposition chronique. En effet, 60% des données sur les effets actuellement disponibles et contribuant à la détermination de ces seuils sont issues de scénarios d'exposition aiguë. Or, dans l'environnement, les espèces se retrouvent exposées de manière chronique, à de faibles doses, et sur le long terme (i.e., plusieurs générations). Peu d'études à ce jour s'intéressent en particulier aux effets multi- et transgénérationnels, quand bien même il a été montré que les générations suivantes sont plus radiosensibles que les premières générations exposées, faisant ainsi baisser le seuil de tolérance aux rayonnements.

Dans ce contexte, la recherche s'intéresse de plus en plus à la détermination des effets à l'échelle sub-individuelle. Dans le cadre de l'évaluation du risque chimique et pour faciliter l'évaluation de la toxicité des milliers de substances mises sur le marché chaque année (règlement REACH), l'OCDE (Organisation de Coopération et Développement Economique) recommande l'utilisation d'approches de criblage (comme les QSAR) permettant de regrouper les substances toxiques par propriétés structurales, leur interaction avec des cibles biologiques, etc. En cohérence avec ces approches, le concept d'AOP (Adverse Outcome Pathway) a été formalisé en 2010, et permet d'identifier des voies de réponses communes entre les toxiques et entre les espèces notamment. (Ankley et al., 2010). Ce concept vise à établir les liens de causalité entre les effets observés à différentes échelles du vivant. Partant d'un évènement moléculaire initiateur (e.g., interaction d'un polluant avec un ligand biologique) et allant jusqu'à un effet adverse à l'échelle individuelle (e.g., diminution de la reproduction) voire des populations (e.g., diminution de la taille de population), les évènements intermédiaires sont séquentiels et précisément reliés par des liens de causalité. Ainsi, par définition, les AOPs se veulent indépendantes du polluant, puisque seuls les effets biologiques sont caractérisés. De fait, ce concept initialement pensé pour l'évaluation du risque chimique peut très bien s'appliquer à l'évaluation du risque radiologique. En plus de sa capacité à structurer de manière relativement simple la séquence d'évènements induisant un effet adverse, ce concept présente la vertu d'agrèger les données à travers une base de données en libre accès (AOP wiki).

Cette thèse s'inscrit dans ce contexte et a pour but de caractériser les effets moléculaires d'une exposition chronique aux rayonnements ionisants chez le nématode *Caenorhabditis*

elegans. Chez les invertébrés, et notamment *C. elegans*, la reproduction, paramètre important au regard de la dynamique des populations, s'est avérée être le paramètre individuel le plus radiosensible. En effet, au niveau individuel, on observe une diminution de la taille de ponte totale d'environ 30% à une dose totale de 3.3 Gy (débit de dose 50 mGy.h⁻¹), sans effet sur la viabilité des embryons qui éclosent normalement (Dubois et al., 2018). Les thèses précédentes se sont attelées à mieux comprendre les mécanismes sous-jacents à cette reprotoxicité et les doses auxquelles ils surviennent, au niveau cellulaire et moléculaire (Buisset-Goussen, 2014, Maremonti et al., 2020b), et en particulier au niveau du protéome (Dubois, 2017, Kuzmic, 2018), du transcriptome ainsi que sur plusieurs générations (Guédon et al., 2021). Au niveau moléculaire, elles ont pu mettre en évidence une dérégulation de certaines protéines et gènes en lien avec la reproduction (développement embryonnaire, maturation des ovocytes, spermatogenèse), le métabolisme des lipides (catabolisme des lipides, surexpression des vitellogénines, protéines transporteur des lipides) et le cycle cellulaire (réplication de l'ADN, apoptose) (Dubois et al., 2019, Buisset-Goussen et al., 2014, Maremonti et al., 2019, Guédon et al., 2021). Au niveau cellulaire, une diminution du nombre de spermatozoïdes ainsi qu'un arrêt du cycle cellulaire en mitose et une augmentation de l'apoptose dans la lignée germinale ont été observés (Buisset-Goussen et al., 2014, Maremonti et al., 2019). Cette thèse s'est donc intéressée à déterminer plus précisément les mécanismes de réponse identifiés ainsi que leurs liens de causalité à différentes échelles. En particulier elle s'est focalisée sur le rôle des lipides en lien avec la reproduction et la cause de la diminution du nombre de spermatozoïdes. Pour affiner la compréhension de ces mécanismes et mieux comprendre leur occurrence au cours du développement de l'organisme, une approche par irradiation en fonction des stades de vie a été adoptée. Ce travail s'articule donc en trois axes de recherche :

- 1) Quel stade de vie est le plus radiosensible vis-à-vis de la reproduction ?
- 2) Quelle est le lien entre le métabolisme des lipides et la diminution de la reproduction observée après irradiation ? Comment contribue-t-il à cette diminution ?
- 3) Comment le développement de la lignée germinale (gamétogenèse) est impacté après irradiation ? Quelles sont précisément les voies de régulation impactées et comment contribuent-elles à la diminution de la reproduction ?

Pour caractériser la radiosensibilité des stades de développement chez *C. elegans*, trois stades d'intérêt vis-à-vis de la reproduction ont été identifiés sur la base de la littérature existante (embryogenèse, gonadogenèse précoce, développement complet). La majorité des expérimentations dans cette thèse ont donc été faite suivant ces trois scénarios d'exposition chronique.

Dans un premier temps, des analyses au niveau de la ponte (taille de ponte, succès d'éclosion, cinétique de ponte), du nombre de spermatozoïdes et au niveau du métabolisme des lipides (lipides neutres, lipides polaires, acides gras) ont été réalisées. Elles ont mis en évidence le fait que les effets reprotoxiques dépendent du stade de développement. L'irradiation au cours de l'embryogenèse conduit à une légère diminution de la taille de ponte, tandis que cette diminution est accrue après irradiation pendant tout le développement et s'accompagne d'effets supplémentaires (diminution du nombre de spermatozoïdes et de la cinétique de ponte). Des effets dépendant du stade ont aussi été observés au niveau des lipides avec une régulation opposée des lipides neutres et polaires en fonction du stade irradié. A partir des connaissances existantes sur la régulation du métabolisme lipidique par la lignée germinale et la lignée somatique chez *C. elegans*, nos résultats ont permis de proposer un modèle conceptuel décrivant le lien entre métabolisme des lipides et la lignée germinale en réponse aux rayonnements ionisants. Ces travaux ont conduit à la rédaction d'un article publié (see Paper I p.86).

Dans un second temps, la troisième question de recherche a été traitée en s'intéressant de manière plus sélective à certaines voies de régulation. Sur la base de la littérature, des voies à la fois impliquées dans la réponse au stress et le développement de la lignée germinale ont été identifiées et étudiées après irradiation selon les mêmes scénarios d'exposition que précédemment. Il s'agissait plus particulièrement des voies de détermination sexuelle via TRA-1 (i.e., la spécification du développement mâle ou femelle de la lignée germinale et gonade somatique), de la voie apoptotique, et de la voie Ras/ERK (MPK-1) impliquée dans de nombreux processus développementaux comme la progression en méiose des cellules germinales, l'apoptose et notamment la transition spermatogenèse/ovogenèse. L'étude de l'apoptose par l'utilisation de mutants a permis de révéler que l'apoptose est responsable de la diminution du taux d'ovulation, qui contribue à hauteur d'environ 10% de la diminution de

la ponte totale. Les spermatozoïdes ne sont pas affectés par l'apoptose et leur diminution, encore non expliquée, est responsable d'environ 90% de la diminution de la ponte après irradiation pendant tout le développement. L'étude de l'expression de MPK-1 dans la lignée germinale a permis de mettre en évidence une suractivation de cette protéine dans la gonade après irradiation probablement liée à une augmentation de l'apoptose radio-induite. Au niveau de la détermination sexuelle, l'étude d'un transgène de la protéine TRA-1 n'a pas permis de révéler une différence d'expression de cette protéine dans l'organisme après irradiation. Ces analyses n'ont pas permis d'expliquer la cause de la diminution du nombre de spermatozoïdes, mais ont permis d'écarter certaines hypothèses et de mieux comprendre la contribution relative des gamètes mâles et femelles chez l'hermaphrodite dans la diminution de la reproduction après irradiation. Ces études font l'objet d'un deuxième article en cours de révision par les co-auteurs (see Paper II p.106).

Finalement, l'ensemble de ces données ont été compilées et synthétisées dans un schéma AOP, de manière à établir les liens de causalité entre les effets et d'identifier les manques de connaissances restantes.

En parallèle de ces travaux de recherche, une réflexion approfondie autour de l'application du concept AOP dans le cadre de l'évaluation du risque radio écologique environnemental a été menée. Le but était d'identifier les défis à relever pour l'application de ce concept dans le contexte de la radioécologie (e.g., spécificité du stresser (mode d'action), durée d'exposition (effets à long terme, chroniques), prise en compte des effets à l'échelle des populations et de l'écosystème...etc.), et de proposer des outils et méthodologies pour les relever.

Ce travail s'articule donc en cinq parties :

- La première partie constitue une introduction générale posant le contexte de cette thèse.
- La deuxième partie correspond à une synthèse bibliographique des différentes thématiques abordées dans la thèse. Elle vise à présenter (i) les connaissances de base sur les rayonnements ionisants et leurs effets biologiques, (ii) le cadre actuel de l'évaluation du risque radiologique dans l'environnement et les défis à relever, (iii) la revue en cours de rédaction sur le concept AOP appliqué dans ce contexte et les challenges associés, (iv) le choix de l'espèce modèle *C. elegans*, ses caractéristiques

anatomiques, physiologiques et moléculaires et leur implication dans la réponse au stress et aux rayonnements ionisants plus spécifiquement.

- La troisième partie traite des objectifs de ce travail de thèse et de la stratégie expérimentale mise en place au cours de ce projet.
- La quatrième partie traite des résultats obtenus et présente une brève synthèse des articles publiés ou en cours de finalisation, ainsi que le schéma AOP construit à partir de ces données.
- La cinquième partie constitue une discussion détaillée de l'ensemble des résultats obtenus au regard de l'objectif initial, ainsi que les perspectives suggérées pour aller plus loin.

En fin de document, les articles scientifiques publiés au cours de cette thèse ou en cours de finalisation sont disponibles

ABSTRACT

Wildlife is chronically exposed to various sources of ionizing radiations, either environmental or anthropic due to nuclear energy use, which can induce several defects in organisms. In invertebrates, reproduction, which directly impacts population dynamics, was found to be the most radiosensitive individual endpoint. Understanding the underlying molecular pathways inducing this reproduction decrease can help to characterize early effects and to understand species radiosensitivity. This type of data can be structured through the AOP (Adverse Outcome Pathway) framework in order to causally link molecular initiating events to adverse outcomes at different biological levels of organization and to compare with other toxicants (exposome). This thesis aimed to decipher the molecular determinants of reproduction decrease (broodsize decrease, no observed hatching defect) in the hermaphroditic soil invertebrate *Caenorhabditis elegans*, after chronic exposure to gamma radiations. Several pathways and biological processes were investigated (lipid metabolism, sex determination and stress-response) to determine the molecular initiating- and key- event(s) of observed reproduction decrease. A life-stage dependent approach was adopted to identify the timing of occurrence and early mechanisms of reprotoxic effects. Our results showed that reprotoxic effects are life-stage dependent and intertwined with the regulation of lipid metabolism by the germline and the soma. Moreover, we showed how both gametes (spermatozoa and oocytes) are affected by different radio-induced response pathways and unevenly contribute to the broodsize decrease. These results will contribute to build an AOP scheme, to causally link the observed effects leading to reproduction decrease, and eventually improve environmental risk assessment.

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COMMON ABBREVIATIONS LIST

AO: Adverse Outcome

AOP: Adverse Outcome Pathway

DTC: Distal Tip Cell

ED/EDR 10/50: Effective Dose/Dose Rate observed for 10/50% of individuals

ERA: Environmental Risk Assessment

GSC/GC: Germ Stem Cells/Germ Cells

IAEA: International Atomic Energy Agency

ICRP: International Commission on Radiological Protection

IR: Ionizing Radiation

KE: Key Event

LET: Linear Energy Transfer

LOED/DR: Lowest Observed Effect Dose/Dose Rate

MIE: Molecular Initiating Event

NORM: Naturally Occurring Radioactive Material

OECD: Organisation for Economic Co-operation and Development

RAP: Reference Animals and Plants

RO: Representative Organisms

ROS: Reactive Oxygen Species

UNSCEAR: United Nations Scientific Committee on the Effects of Atomic Radiation

1 INTRODUCTION

In the environment, wildlife is chronically exposed to various sources of ionizing radiation, whether environmental or anthropogenic due to the use of nuclear energy, which can cause a variety of defects in organisms.

Historically, environmental risk assessment was assumed to be covered by human radiation protection, postulating that since humans are the most sensitive species, their protection would include the protection of all species. However, the issue of biodiversity loss and the importance of protecting the environment as such have gained attention, notably through the Convention on Biological Diversity and the Rio Declaration during the United Nations Conference on Environment and Development in 1992 (UN, 1992b, UN, 1992a). Since then, new criteria have been and continue to be established to ensure better risk assessment in non-human species.

In the context of environmental radiological risk assessment (ERA), an essential step in protecting species is the ability to determine toxicity thresholds (i.e., reference values) below which no effects are observed in species. However, these thresholds were based on available data that is not always representative of environmental situations (type of exposure, representative species, interactions with environmental factors, etc.). In this context, there is a growing need to acquire more data (e.g., chronic exposure, multigenerational data, field data, etc.) to refine these thresholds. Moreover, the radiosensitivity of species is distributed across a wide range of dose rates, which is not fully explained. The acquisition of mechanistic information (i.e., sub-individual data) could help us better understand the determinants of these species-specific sensitivities. However, this type of data is difficult to integrate into the ERA framework, which focuses on individual effects. Thus, causal links need to be established between mechanistic information and phenotypic responses to increase predictivity.

In the context of chemical risk assessment and to facilitate the evaluation of the toxicity of thousands of substances placed on the market each year (REACH regulation), the OECD (Organization for Economic Cooperation and Development) recommends the use of screening approaches (e.g., QSAR) to group substances by structural properties, interaction with

biological targets, etc. In line with this approach, the concept of AOP (Adverse Outcome Pathway) was formalized in 2010, to find common pathways between toxicants, species, etc. (Ankley et al., 2010). This concept aims to establish the causal links between the observed effects at different biological scales. Starting from a molecular initiating event (e.g., interaction of a pollutant with a biological ligand) leading to an adverse outcome at the individual scale (e.g., decrease in reproduction) or even at the population scale (e.g., decrease in population size), the intermediate key events are sequential and precisely connected by causal links. Thus, by definition, AOPs are intended to be independent of the pollutant, since only biological effects are characterized. In fact, this concept initially designed for chemical risk assessment can be applied to radiological risk assessment.

In this context, this thesis aims to characterize the molecular effects of chronic exposure to ionizing radiation in a particular species, the nematode *Caenorhabditis elegans*. In invertebrates, including *C. elegans*, reproduction, an important parameter for population dynamics, has been shown to be the most radiosensitive individual parameter. Indeed, a decrease in total broodsize of approximately 30% with no effect on the viability of hatching embryos was previously found at the lowest total dose of 3.3 Gy (dose rate of 50 mGy.h⁻¹) (Dubois et al., 2018). Previous theses have worked to better understand the mechanisms underlying this reprotoxicity, at the cellular and molecular levels (Buisset-Goussen, 2014, Maremonti et al., 2020b), and on the proteome (Dubois, 2017, Kuzmic, 2018), transcriptome as well as over several generations (Guédon et al., 2021). At the molecular level, they highlighted a deregulation of certain proteins and genes related to reproduction (embryonic development, oocyte maturation, spermatogenesis), lipid metabolism (lipid catabolism, overexpression of vitellogenins, lipid transporter proteins) and cell cycle (DNA replication, apoptosis) (Dubois et al., 2019, Buisset-Goussen et al., 2014, Maremonti et al., 2019, Guédon et al., 2021). Interestingly, it was shown that chronic and acute responses on reproduction endpoints (hatching success) were different, in particular considering the cellular defense response, proteomic response and threshold doses (Dubois et al., 2018). This highlights the importance of acquiring more data after chronic exposure that cannot be extrapolated from acute exposure data. At the cellular level, a decrease in sperm count as well as cell cycle arrest in mitosis and increased apoptosis in the germline were observed (Buisset-Goussen et al.,

2014, Maremonti et al., 2019). This thesis was therefore interested in further investigating these mechanisms. In particular, we focused on determining a more radiosensitive life-stage regarding reproduction decrease. The goal was to better understand the timing of the occurrence of these mechanisms during the development of the organism. Then, we focused on the role of lipids in relation to reproduction and the cause(s) of sperm decrease. This work is thus articulated around three research questions:

- Question 1: Is there a more radiosensitive life stage regarding reproductive outcome?
- Question 2: What is the connection between lipids and reproduction in response to ionizing radiation? How do they contribute to the decrease in reproduction?
- Question 3: Are developmental pathways in the germline involved in the response to ionizing radiation? How do they contribute to the decrease in reproduction?

To characterize the radiosensitivity of developmental stages in *C. elegans*, three stages of interest with respect to reproduction were identified based on the existing literature (embryogenesis, early gonadogenesis, full development). Most of the experiments in this thesis were therefore performed according to three chronic exposure scenarios corresponding to these developmental stages. All the data obtained was integrated into an AOP scheme to establish causal relationships between observed effects and increase their predictive potential.

This work is presented in five parts:

- The first part is the present general introduction.
- The second part corresponds to a bibliographic synthesis of the various themes addressed in the thesis. It aims to present (i) basic knowledge on ionizing radiation and its biological effects, (ii) the current framework of radiological risk assessment in the environment and the challenges to be met, (iii) the review (in progress) on the application and challenges of the AOP concept applied in the radioecology context, (iv) the choice of the model species *C. elegans*, and its anatomical, physiological and molecular characteristics and their implication in the response to stress and ionizing radiation more specifically.

- The third part deals with the objectives of this thesis and the experimental strategy implemented during this project.
- The fourth part deals with the results obtained and presents a brief summary of the articles published or in the process of being finalized, as well as the AOP scheme built from the present data.
- The fifth part constitutes a detailed discussion of all the results obtained with respect to the initial objective and the perspectives and knowledge gaps that remain.

Finally, at the end of the document, the scientific articles published during this thesis work are made available.

2 STATE OF THE ART AND CHALLENGES

2.2 IONIZING RADIATION: DEFINITION AND BIOLOGICAL EFFECTS

2.2.1 Origin and nature of ionizing radiation

Radioactivity refers to the emission of particles or electromagnetic rays by an unstable atomic nucleus (e.g., due to an excessive number of neutrons or protons) which attempts to reach a stable state. Radioactivity could be natural or artificial due to anthropic activities. This phenomenon, called radioactive *decay* since the number of unstable nuclei *decreases* as it occurs, releases energy in the form of either particles (e.g., α , β) or electromagnetic rays (e.g., UV, X, γ), depending on the nature of the radionuclide. In particular, α , β , γ or X rays are said to be 'ionizing' because they release sufficient energy to ionize molecules, (i.e., removing an electron from an atom). This can profoundly change the structure and therefore the function of a biomolecule (i.e., water, lipids, DNA, proteins, etc.). The extent of damage in biological tissues is directly dependent on the nature of radiation, the energy deposited by this radiation and its trajectory. This is defined by the linear energy transfer (LET), which is the amount of energy released to the material traversed per unit distance ($\text{eV}\cdot\mu\text{m}^{-1}$). While α particles have a high LET (meaning they transfer most of their energy in short distances), β and γ rays have lower LET. A lower LET means that these types of rays are capable of penetrating biological tissues more deeply, although they deposit less energy along their track (Figure 1). To quantify the exposure of an organism/tissue to ionizing radiation, the basic quantity is the energy absorbed by unit mass of tissue of an organ or an organism (J/kg); given in grays (Gy) (ICRP, 2008).

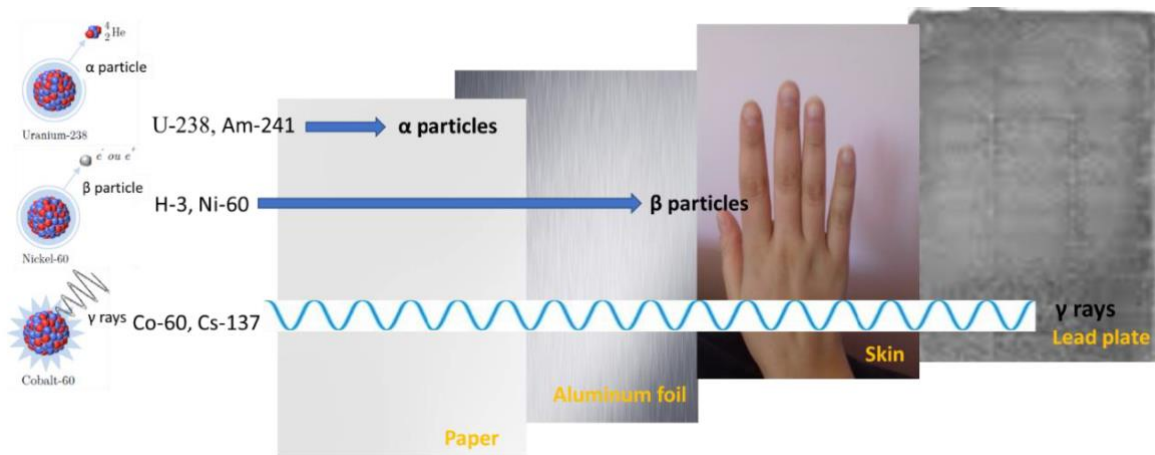


Figure 1 Penetrating power of different types of ionizing radiation (Kuzmic, 2018)

- Radiation in the environment

In the environment, exposure to radiation can originate from several sources. Natural sources of radiation (e.g., cosmic rays or telluric radioactive material) are responsible for a mean dose rate of $3.11 \text{ mGy}\cdot\text{year}^{-1}$ in terrestrial organisms (Bey and Meador, 2011), and $0.02\text{--}2.6 \text{ mGy}\cdot\text{year}^{-1}$ in aquatic organisms (Hoffman et al., 2002). These Naturally Occurring Radioactive Materials (NORM) (e.g., α emitters radon, thorium, uranium) can be found in more or less important concentrations depending on geological and geographical context. Also, these NORMs comprise the raw material for the nuclear industry, and their extraction and exploitation can increase exposure risk by generating new radionuclides (e.g., Cs-137 obtained from the fission of U-235) and locally increasing concentrations. In addition, the use of other radionuclides in the medical field (mostly Technetium-99m and Iodine-131) have also increased exposure risk of populations (e.g., via CT scans, X rays, etc.), as well as their release in the environment. The extended use of nuclear science in several sectors (medical, energy, military) have thus led to an increased risk of exposure of environmental biota. However, releases from the nuclear industry are minimal and regulated in France and thus appear to be of less concern compared to accidental releases, even if the latter are less probable. For example, in France, the dose received by aquatic organisms due to controlled releases from nuclear plants is about $0.01 \text{ mGy}\cdot\text{year}^{-1}$ (IRSN, 2021). In contrast, the dose rates found in the environment of the Chernobyl nuclear power plant 20 days after the 1986 accident reached $20,000 \text{ mGy}\cdot\text{d}^{-1}$ (UNSCEAR, 2008). In spite of radioactive decay, the residual radioactivity in the Chernobyl Exclusion Zone still reached $0.0024 \text{ mGy}\cdot\text{d}^{-1}$ to $24 \text{ mGy}\cdot\text{d}^{-1}$ in the last decade

(Garnier-Laplace et al., 2013). These external dose rates are notably due to the presence of persistent Cs-137 (γ), Sr-90 (β) and Pu-239 (α). In comparison, the measured dose rate in Fukushima-Daiichi 30 days after the accident in 2011 was about 2600 mGy.d⁻¹ in the benthic biota (most exposed ecosystem in this situation)(Garnier-Laplace et al., 2011).

- Exposure pathways

In the environment, radionuclides can induce harmful effects to organisms either through external exposure or internal contamination (ingestion, inhalation, contact). α and β emitters are thus very minimally harmful through external exposure, but much more so in the case of internal contamination because of their high LET (Figure 1). In contrast, γ emitters are harmful even through external exposure and can penetrate as deep as the cell nucleus (Delacroix et al., 2006). In addition, radionuclides are intrinsically both radiotoxic and chemotoxic. Thus, when internalized, their chemotoxicity must also be taken into account when assessing environmental risk.

2.2.2 Radioinduced biological effects

Ionizing radiation can alter biological pathways, either via direct or indirect effects. Direct effects occur when molecules are directly ionized by radiation, while indirect effects are caused by the production of free radicals and Reactive Oxygen Species (ROS) in cells *via* water ionization and radiolysis. Because water constitutes 80% of the cell, it is the primary target of ionizing radiation (Draganic, 1971), but it can also reach other biomolecules such as DNA, lipids and proteins. A brief overview is given in the following section.

- Oxidative damage and ROS production

Several components are generated upon water radiolysis such as free radicals (e.g., O₂^{•-}, HO[•] and ROS (e.g., H₂O₂)) which are characterized by higher activity than molecular oxygen (Laverne and Shuler, 1983, Laverne, 1989, Meesungnoen et al., 2001). The amount of ROS generated from ionization leads to the further propagation of endogenous ROS via the perturbation of ROS-producing systems (e.g., mitochondria) that induces additional perturbation (Choi et al., 2007, Kam and Banati, 2013). Then, the resulting oxidative damage of cells and tissues is further propagated due to the interaction between ROS and other biomolecules (e.g., DNA, lipids, proteins), leading to the imbalance of cellular redox status.

Although other macromolecules can also be affected by direct ionization, their alteration by indirect effects is more described. The most known effect on lipids is lipid peroxidation, which can disturb the integrity of cellular membranes, and thus homeostasis of cells (Stark, 1991, Hammer and Wills, 1979). Products resulting from lipid peroxidation are generally stable, toxic and likely to react with other biological components, increasing the initial damage from free radicals (Ayala et al., 2014). Antioxidant systems can protect cells from ROS-induced lipid peroxidation such as enzymatic antioxidants (e.g., Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx)) and non-enzymatic antioxidants (e.g., vitamins, minerals such as selenium and zinc, etc.)(Su et al., 2019). Products of lipid peroxidation (e.g., 4-Hydroxynonenal (4-HNE)) can mediate the autophagy (i.e., the process by which macromolecular substances are degraded in cells) through several pathways (Ayala et al., 2014) and lead to another form of cell death called ferroptosis (Xie et al., 2020).

Proteins are altered by modification of amino acids (depletion, oxidization, substitution, etc.), thus modifying their secondary and tertiary structures and leading to different types of damage and unfolding (Houée-Levin and Bobrowski, 2013, Reisz et al., 2014). If proteins are too damaged, either by direct damage or oxidative stress, they can be degraded by specific repair systems such as the proteasome (Dubois et al., 2018, Dubois, 2017, McBride et al., 2003).

DNA damage (either direct or indirect) has extensively been studied because of the importance of this molecule for the integrity of cells and inheritability of genetic material across generations. There are many types of DNA damage and they are more or less complex, depending on the nature of the lesion and reparability (e.g., base damage, deoxyribose backbone damage, DNA chain breaks (single (SSBs) or double strand breaks (DSBs)) (Ward, 1994, Duncan Lyngdoh and Schaefer, 2009, Thompson, 2012). In response to these types of damage, cells have DNA damage checkpoints to trigger different repair mechanisms according to the cell type (e.g., somatic or germinal) and the cell cycle (e.g., proliferating vs differentiated) (Clejan et al., 2006, Sakashita et al., 2010, Cannan and Pederson, 2016, Chatterjee and Walker, 2017). Thus, cell fate will depend on these parameters and the cell-specific radiosensitivity will also directly impact life-stage sensitivity in organisms, as

precocious life stages exhibit numerous cells in rapid proliferative state (Adam-Guillermin et al., 2018).

- Cell fates

The accumulation of oxidative stress in addition to direct damage of these biomolecules will lead to a variety of cell damage responses, such as cell cycle arrest, altered proliferation, mutations, apoptosis, membrane rupture, distorted signalling networks and mitochondrial dysfunctions (Spitz et al., 2004, Azzam et al., 2012). Figure 2 summarizes a few scenarios. From a few minutes to a few days after exposure, if repair mechanisms are saturated, they might not be able to properly repair damages to molecules, inducing elimination of the cell either by necrosis or programmed cell death, i.e., apoptosis. However, in the case of non-lethal mutation, the cell might survive but with mutations that can either be transmitted to the next generations if they are germ cells or lead to uncontrolled proliferation if they are somatic cells. Effects on germ cells are of particular interest in radioecology since they might affect fecundity and/or fertility of organisms and thus population dynamics in the long term.

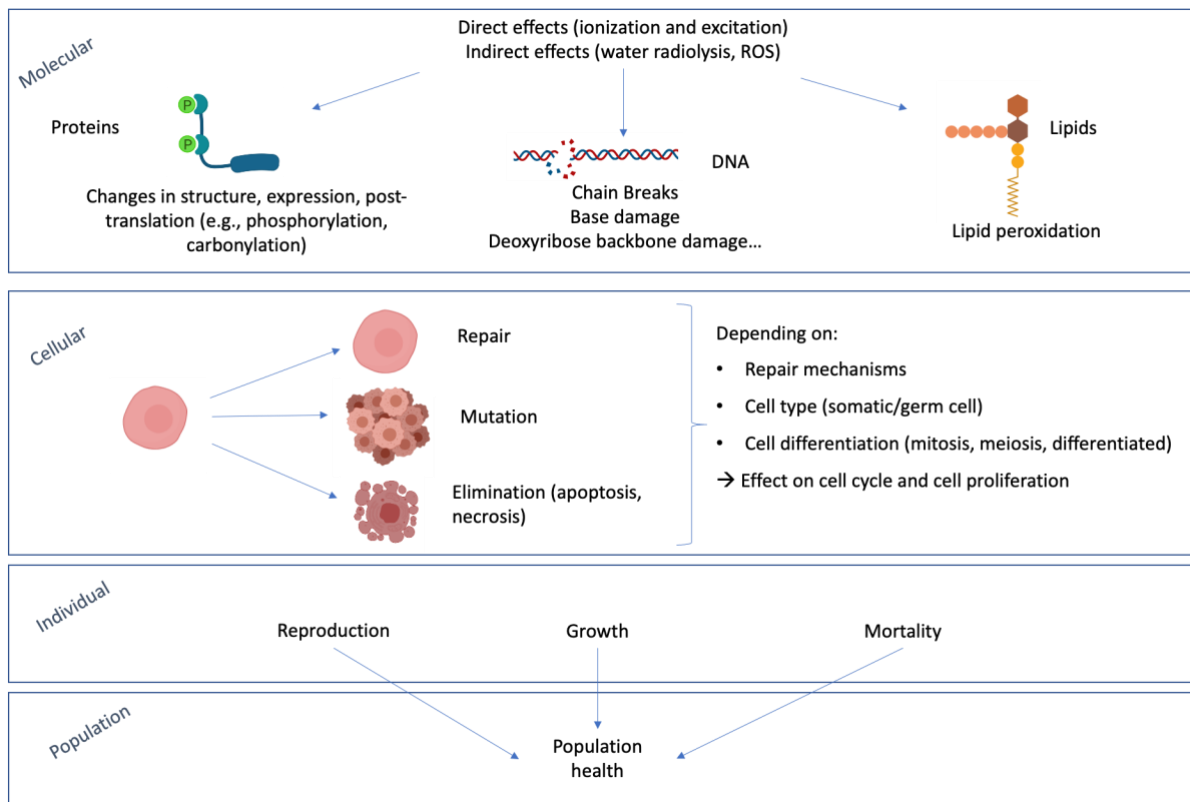


Figure 2 Summary of biological effects of ionizing radiation at different biological levels of organization.

- Contribution of indirect and direct effects according to radiation type

For low LET radiation (e.g., γ rays including Cs-137, used in this project), indirect effect through radiolysis of water is the predominant process (70% vs 30% of direct effects), while for high LET radiation, direct effects are more preponderant (Friedland et al., 2017, Kundrat et al., 2020, Urushibara et al., 2008). Furthermore, secondary particles produced along the track of high LET particles are usually low LET radiation. Thus, high-LET radiation combines tracks from high- (primary) and low- (secondary – particles) LET radiation, which may considerably modulate the biological response.

2.3 RADIOECOLOGICAL RISK ASSESSMENT

2.3.1 Regulatory framework and stakeholders

Several international organizations exist to provide either recommendations or safety requirements on the use of nuclear energy, based on scientific knowledge acquired worldwide.

The UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation) issues public reports on Sources and Effects of Ionizing Radiation that serve as a review of scientific knowledge needed for future recommendations. From these reports, the ICRP (International Commission on Radiological Protection) provides recommendations that form the basis of radiological protection policy, regulations, guidelines and practice worldwide. Finally, the IAEA (International Atomic Energy Agency) issues safety standards either as guides or requirements that are used as a legal basis for governments and decision makers (Figure 3).

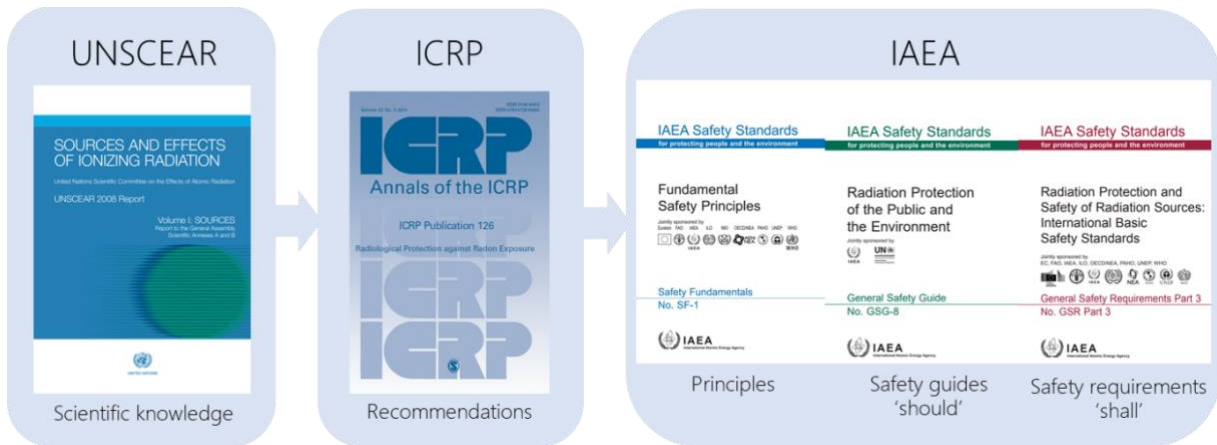


Figure 3 Diagram of principal stakeholders, roles and relationships (adapted from (IRSN, 2016))

In France, the ASN (Authority of Nuclear Safety) acts nationally to ensure nuclear safety and radioprotection of workers, populations and the environment. The IRSN (Institute for Radioprotection and Nuclear Safety) acts as the public expert on nuclear safety and radioprotection and is involved in UNSCEAR reports from its research activities, ICRP activities either for prospective assessment (planned exposure situations) or retrospective assessment (existing exposure situations, accidental exposure) and establishment of safety standards with the IAEA.

2.3.2 Environmental Risk Assessment in Radioecology: Threshold definition and methodology

2.3.2.1 Brief history and definition of threshold values

Until 1991, ecosystems were considered in terms of radioprotection only as a vector of exposure for human health, postulating that the human species is the most radiosensitive, and protecting it would protect all other species (ICRP, 1991). Since the Rio declaration (UN, 1992b) and convention on biological diversity (UN, 1992a), society has been made more aware of the importance of protecting biodiversity and wildlife itself. Since then, the IAEA and UNSCEAR started to propose reference values specific to non-human species (Whicker and Bedford, 1995). In 2008, the UNSCEAR proposed “...chronic dose rate of $100 \mu\text{Gy}\cdot\text{h}^{-1}$ ($2,4 \text{ mGy}\cdot\text{d}^{-1}$) to the most highly exposed individuals unlikely to have significant effects on most terrestrial animal populations” or “the maximum dose rates of $400 \mu\text{Gy}\cdot\text{h}^{-1}$ ($9,6 \text{ mGy}\cdot\text{d}^{-1}$) to a small proportion of the individuals in aquatic populations of organisms that would not have any detrimental effect at the population level” (UNSCEAR, 2008). Other reference values have

since been proposed due to the development of European research projects (such as FASSET¹ and ERICA² projects)(Howard and Larsson, 2008, Larsson, 2004) and growing databases such as the FREDERICA³ database (Copplesstone et al., 2008). This database is filled with more than 32,000 radiotoxicity data for 14 taxonomic sub-groups (bacteria, algae, plants, insects, molluscs, crustaceans, zooplankton, amphibians, birds, fish, mammals, soil invertebrates, fungi, mosses and lichens), grouped in four categories of effects: mortality, morbidity, reproduction and mutation. This data, collected from the literature, was used to derive dose-response relationships for each species, and thus threshold values such as ED₅₀ (Effective Dose, i.e., the dose at which an effect is observed for 50% of individuals for acute exposure) or EDR₁₀ (Effective Dose Rate for 10% of individuals for chronic exposure). These data can then be used to model Species Sensitivity Distribution (SSD) which is then used to derive a unique threshold value for protection of ecosystems. This approach was used to determine the reference value of 10 $\mu\text{Gy}\cdot\text{h}^{-1}$ for protection of generic ecosystems (Garnier-Laplace et al., 2010) (Figure 4). However, the methodology for establishment of SSD is not always appropriate (selection of species, model used to fit the distribution, type of exposure), and thus the values extrapolated from this SSD should be considered with caution (Spurgeon et al., 2020).

¹ Framework for assessment of environmental impact of ionizing radiation in European ecosystems

² Environmental Risk from Ionising Contaminants: Assessment and Management

³ [Frederica Database \(frederica-online.org\)](http://frederica-online.org)

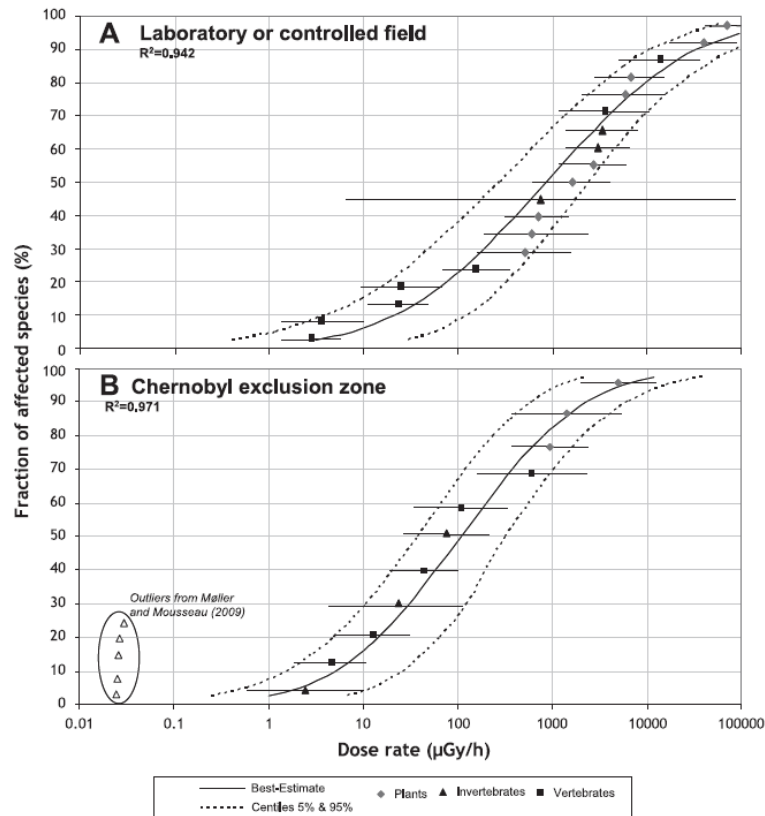


Figure 4 Extrapolation approach proposed for modelling interspecies radiosensitivity variation. (A) species sensitivity distribution (SSD) fitted to minimum values of EDR10 (dose rate giving 10% change in observed effect) for terrestrial species exposed under controlled conditions to external gamma irradiation; (B) SSD fitted on EDR10 data set acquired in real field conditions from the Chernobyl Exclusion Zone (Garnier-Laplace et al., 2013).

Many studies, particularly within the ERICA project, currently use this value as a reference value for the protection of ecosystems. However, the choice of reference values might vary from one country to another based on the methodology and the definition of the object of protection (ADAM C. et al., 2006). For example, the NCRP (US National Council on Radiation Protection and Measurements), UNSCEAR and IAEA have estimated a threshold protection value of 400 $\mu\text{Gy}\cdot\text{h}^{-1}$ (NCRP, 1991, IAEA, 1992, Larsson, 2008, UNSCEAR, 2008). Other approaches such as the ICRP approach recommend the use of Reference Animals and Plants (RAPs) representative of different taxa, to establish derived consideration reference levels (DCRLs), which are bands of dose rate where some sort of detrimental effect in a particular RAP may be expected to occur following chronic, long-term radiation exposure (ICRP, 2008). Although methodologies differ, most have highlighted the fact that protection values are obtained from data somehow lacking ecological relevance given mode of exposure,

dose or dose-rate or species used (Spurgeon et al., 2020). Within the current methodological framework of radioprotection of the environment, these reference values are used to conduct the analysis of effects, which, in combination with analysis of exposure, aim to precisely characterize the risk to ecosystems.

2.3.2.2 Methodology of Environmental Risk Assessment: the ICRP approach

Nowadays, the framework for Environmental Risk Assessment (ERA) in radioecology is well established. The following part focuses on the ICRP approach as it is the most used worldwide because of its ease of application and the ICRP international status. The method for assessing radiological risk to wildlife is based, like the one for chemical risk, on the principle of "deviation from reference", i.e., the comparison of a level of exposure of organisms to radionuclides in relation to a reference (i.e., previously explained threshold values). As for human radiation protection, exposure is quantified by a dose to organisms, calculated as a function of a radionuclide source (releases), transfer and exposure pathways, characteristics of exposed organisms, and the exposure scenario (behaviour, ecological parameters) (K. Beaugelin-Seiller et al., 2021).

It consists of a 3-tiered approach from most conservative to most realistic scenario. Thus, advancing through the tiers will require more data and resources. For each tier, 4 components are considered: the context of assessment, analysis of exposure, analysis of effects and risk characterization. Risk characterization combines both exposure level and effects in order to quantify the probability of a risk for the species to protect. Analysis of exposure aims to quantify the levels of exposure of organisms from all types of exposure, by collecting field or modelled data when measures are not available. Two types of data are necessary: concentration factors (CFs), unless measures in organisms are already available, and Dose Coefficients (DCs).

CFs are a quantification of the transfer of radionuclides to organisms from diverse vectors of exposure (e.g., food, water, soil). Many values of CFs can be found on the Wildlife Transfer Database⁴ online, among other sources of data. In the database, they were collected for 40 taxonomic groups, belonging to 3 ecosystems (terrestrial, marine and freshwater) and for about 70 radionuclides associated with diverse sources (NORM, routine discharges, accidents

⁴ [Wildlife Transfer Parameter Database \(wildlifetransferdatabase.org\)](http://wildlifetransferdatabase.org)

and waste storage). However, there is a significant uncertainty weighing on CFs. The Wildlife transfer database shows a significant lack of relevant data from numerous radionuclides, limited geographical coverage of data (mostly limited to temperate climate) and the dominance of the adult life stage of organisms, even though it has been showed that precocious life stages can be more sensitive to ionizing radiation (Adam-Guillermin et al., 2018).

DCs are *'coefficients relating an absorbed dose rate in the whole body, or in a part of it, and radionuclide activity concentration in the body for internal exposure, or in the environment in the case of external exposures'* (ICRP, 2017). Since radioecology faces the challenge of relating exposure to dose and dose to effects, the ICRP has suggested the use of 12 different Reference Animals and Plants (RAPs) based on a dosimetric approach and representative of a given ecosystem (ICRP, 2008), from which the DCs are calculated. RAPs are defined as *'a hypothetical entity, with the assumed basic characteristics of a specific type of animal or plant, as described to the generality of the taxonomic level of Family, with defined anatomical, physiological, and life-history properties, that can be used for the purposes of relating exposure to dose, and dose to effects, for that type of living organism'* (ICRP, 2008). However, species that are not *a priori* characterized by this dosimetric approach are not considered, which is problematic in terms of ecological representativity. Representative Organisms (ROs), introduced by the ICRP in Publication 124 (ICRP, 2014), overcome this issue as they are representative of a contaminated environment and provide a basis for the estimation of radiation dose rate in this specific environment (Brown et al., 2016, Rhodes et al., 2020). In practice, DCs are used to evaluate doses and dose rates, and to compare the latter with DCRLs.

Analysis of effects aims to choose relevant threshold reference values for ERA (i.e., Predicted No Effect Dose Rate – PNEDR for radioactive substances). These threshold values are obtained prior to ERA, as previously described, and for non-human species, most data come from the FREDERICA database.

2.3.3 Knowledge gaps and challenges

Despite the well-established methodology, several knowledge gaps persist either from a lack of data or a lack of tools to integrate specific data into this methodological framework. We focus here on the knowledge gaps and challenges associated with the determination of threshold values, as it is part of the analysis of effects to which this project contributes. To

illustrate the impact of these different knowledge gaps, we focus on the comparison of SSD from Garnier Laplace et al., 2013 (Figure 4) derived from lab data and *in situ* data and highlight the basis of such differences.

- Lack of data regarding exposure situation

In the FREDERICA database, 65% of data come from acute exposure situations (i.e., short period and doses ranging from 0.1Gy to 1000Gy). However, chronic exposure situations, and especially low-doses (i.e., $<100 \mu\text{Gy}\cdot\text{h}^{-1}$ (UNSCEAR, 1996)), are the most common exposure situation in the environment. When conducting analysis of effects for ERA, threshold values in the situation of chronic exposure are often extrapolated from acute data and safety factors are applied to account for extrapolation (Chapman et al., 1998). This extrapolation from acute to chronic is not always relevant, partly because the underlying mechanisms and mode of actions (MoA) are not necessarily the same between acute and chronic effects (Beaugelin-Seiller et al., 2020, Dubois et al., 2018, Pereira et al., 2011). In addition to the differences in MoA, effects on organisms (e.g., individual effects) upon chronic exposure mostly occur at much lower total doses than for acute exposure (see part 'effects of IR on invertebrates'). All these considerations call into question the protection threshold value of $10\mu\text{Gy}\cdot\text{h}^{-1}$. Therefore, there is a growing need to collect data at more realistic exposure scenarios, i.e., dose rates and long-term chronic exposure.

In addition, *in natura*, species are exposed to multiple types of stressors which can interact together to induce synergetic or antagonistic effects, influencing the observed sensitivity in wild species. Aside from other stressors, wild species are constantly exposed to changes in biotic and abiotic factors (temperature, light, salinity, etc.) that can act as confounding factors for observed sensitivity.

- Species representativity and sensitivity

SSDs are established from available data on a large set of species, that do not all have the same impact on ecosystems (e.g., keystone species are not necessarily considered, although they play a critical role in maintaining the structure of an ecological community) (Spurgeon et al., 2020). Moreover, some SSDs use data available from lab species that are well-characterized biologically and for which data acquisition is facilitated. However, they are far

from realistic when it comes to ecological representativity of an ecosystem or environmental conditions.

To better understand the differences between SSDs derived from field data and SSDs from lab data, in addition to collecting more field data and in using more realistic conditions of exposure, the use of molecular information could be of great help.

- Integration of molecular data into the ERA framework as a future challenge

Molecular data can be very useful to determine more sensitive threshold effects, and notably understand species-specific differences in radiosensitivity (Spurgeon et al., 2020).

Current threshold values used for ERA were elaborated from individual parameters (i.e., mortality, morbidity and reproduction) relevant to population dynamics (quantitative aspect of species conservation), but they do not account for the numerous data at the sub-individual level (e.g., genotoxicity) (qualitative aspect of species conservation) which can be key elements for understanding species sensitivity. Indeed, one main issue is the interpretation of molecular data in terms of dose-response relationships to derive them as ecological protection criteria. For ERA, the outcome of interest is at the individual scale and beyond. Thus, causal links need to be established between sub-individual data and phenotypic outcomes. Some new methodologies were developed for this purpose. For example, the AOP (Adverse Outcome Pathways) concept was created to encourage such links. It does not directly provide tools to establish these links, but it provides a framework to gather and structure data together, identify knowledge gaps, assess the weight of evidence of relationships, etc. Tools that help to establish these causal links are, for example, DRomics (Larras et al., 2018), i.e., dose-response relationships on -omics data.

To summarize, the importance of considering (i) species representative of the environment, (ii) at different life stages, (iii) more sensitive parameters (mechanistic information) to better understand radiosensitivity differences among species, and (iv) exposure situations relevant to environmental situations (i.e., chronic, multi-exposure) is well supported. All these limitations represent challenges to be met in order to improve ERA in radiation protection of the environment.

2.4 LINKING EARLY MOLECULAR EVENTS TO INDIVIDUAL RESPONSES: ADVERSE OUTCOME PATHWAYS

In line with the context of this thesis project, we questioned the possibility and the means of integrating the AOP concept in the context of radioecology. This process led to the writing of a review entitled 'Developing AOP in the context of radioecology: challenges and insights', still in progress, involving several researchers who are experts in their field (radiobiology, ecology, ecotoxicology, modelling). Its coordination was part of this thesis work with two other researchers, Sandrine Frelon and Olivier Armant. In this part, we present the introduction of the paper and its main argument and then provide an overview of the different topics developed through the presentation of the detailed table of contents and associated ideas.

Developing AOP in the context of radioecology: challenges and insights

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2.4.1 Introduction

The growing use of pollutants and their releases in the environment have increased the need to better assess effects on wildlife. To this purpose, Adverse Outcome Pathways (AOPs) have been proposed as a tool by the OECD to aid regulatory decision makers in better

describing effects and deriving threshold values to protect the environment (Ankley et al., 2010). AOPs aim to describe a sequence of causally linked events, called Key Events (KE), from the Molecular Initiating Event (MIE) to an Adverse Outcome (AO) at a relevant biological scale, usually individual for human health or population for the Ecological Risk Assessment (ERA). Each key event (KE) is defined by its essentiality, meaning they are necessary to induce the subsequent event. This can be assessed either by direct evidence (e.g., Knock-out of KE1 or early KEs leads to blockage of all downstream KEs) or indirect evidence (e.g., Impact on a modulating factor for early KEs leads to expected pattern of effects on later KEs). Based on the assembled evidence on essentiality for the KEs, confidence in the supporting data on essentiality is considered for the entire AOP, including KERs (Key Event Relationships) and KEs. Confidence in the supporting data for essentiality of KEs, i.e., Weight of Evidence (WoE), within the AOP is considered either low, moderate or high depending on several criteria defined by the OECD guidelines, such as the Bradford-Hill criteria (OECD, 2018, Meek et al., 2014, Becker et al., 2015). Furthermore, each AOP has a defined biological domain of applicability (i.e., sex, life stage, taxa, etc.).

Because AOPs, by definition, are not stressor or species-specific, extraction and synthesis of consensus information from different taxa and chemical domains for construction of models with broad applicability is facilitated. An AOP knowledgebase has been developed through four main components: the AOP-Wiki, Effectopedia, AOPXplorer, and the Intermediate Effects Database (<https://aopkb.oecd.org/index.html>). Any AOP developer can use these databases to help develop an AOP or connect one AOP to others, contributing to the creation of AOP networks. Hence, the potential to share data and sum-up knowledge makes AOPs a great tool to be used in ERA.

AOPs are rather new in the field of radioecotoxicology and few have already been developed (Song et al., 2020, Fay et al., 2017). This is probably because there is little data on subindividual effects of radiation because it was difficult to integrate into the ERA framework of radioprotection until now. The current methodology proposed by the ICRP for radioecological risk assessment requires the characterization of two major components of a classical risk assessment approach: exposure and effects. Regarding characterization of effects, the purpose is to derive threshold reference values based on individual effects such

as reproduction, morbidity and mortality, identified as ecologically relevant. However, sub-individual effects such as genotoxicity data, although particularly significant regarding radiotoxicity, have rarely been considered for ERA, or simply used as complementary data. Indeed, this type of data is difficult to interpret as dose-response relationship, which is needed to integrate them into ecological protection criteria. By definition, the AOP framework qualitatively and quantitatively connects this type of data to ecologically relevant adverse outcomes, by integrating different biological scales. It enables the integration of dose-response relationships into AOPs, making them more quantitative (qAOP) and establishes causally linked events to predict effects at larger scale from MIEs.

They represent a great tool as they allow, for instance:

- Better understanding of toxicants and their integration in several biological pathways: shared AOPs with different toxicants (AOP networks), adding different toxicants culminating in a common AO (addition), explicit interactions between toxicants at some KEs, MIEs
- Species comparison (contrary to AOPs in toxicology for which the species-target is human), allowing for generic and specific AOPs
- Highlighting of the nodal point useful for biomarker identification (as MIE and KE are easily measurable events, they could be used as biomarkers) (Hutchinson et al., 2006), which is useful to derive exposure limit values (qAOP) and apply them in a regulatory context
- Improvement of predictivity of modelling approaches (such as TKTD models) by confirming one mode of action (MoA) rather than another, and then improvement of the prediction of the associated toxic kinetics, therefore reducing uncertainty.

However, there are many challenges and knowledge gaps to overcome when using this tool for radioprotection of the environment. This review aims to present these challenges in a first chapter, and then introduce approaches and how they can be integrated into the AOP concept to overcome these challenges. In the following part, we introduce the main content of this review with a summary of ideas developed in the different sections.

2.4.2 Challenges specific to radioecotoxicology for AOP development

This first section aims to describe the challenges linked either to radionuclides (and their different emitter properties) and exposure of organisms (chronicity, multipollution) or to ecological endpoints themselves (individual, population, etc.).

In the first part, we discuss the **physical properties of radiation** and their interaction with biological media. Even though AOPs are stressor agnostic, the biological effects are highly dependent on the types of radiation. At the molecular level, the distribution of effects, i.e., indirect via water radiolysis or direct effects, depends on the energy and the track structure of radiation (i.e., spatial distribution pattern of ionizations and excitations). This track structure, often assessed by the LET, directly depends on the nature of emitters and quality and energy of ionizing radiation (particle type (alpha, electrons) or electromagnetic type (X, gamma)) (Friedland et al., 2017, Kundrat et al., 2020, Urushibara et al., 2008). Therefore, there is not one unique MIE for radiation but rather different MIEs (e.g., ROS production and direct damage of DNA) that might not be equally distributed depending on radiation type. Subsequently, cell fate (apoptosis, survival, mutations) depends on the absorbed-dose (or -rates), type of ionizing radiation, and spatial pattern of energy deposition (Katz and Cucinotta, 1999, Hall, 2012). All these factors will impact the event cascade leading to different cellular effects.

The second part focuses on the **challenges linked to the integration of more ecologically relevant parameters** in the AOP, referring to exposure situations and the biological level considered (i.e., population, ecosystem). Currently, most available data is on acute exposure situations from which chronic data is often extrapolated. In this part, we show how the extrapolation is not always accurate since the MIE, KE and even AO might differ depending on the mode of exposure, its duration or targeted life stage of the organism considered, (Dubois et al., 2018, Pereira et al., 2011). In addition, organisms in the environment are exposed to multiple types of pollution, that might act in combination to enhance or diminish each other's toxicity. The existing models (such as "Concentration Addition" and "Independent Action" models (Jonker et al., 2004)) that take into consideration these interactions between toxicants are presented, as well as their limits and how AOP framework can be useful to decrease uncertainties and more accurately apply such models.

Another consideration that is challenging for AOPs, but crucial for ERA, is the extrapolation from individual data to population adverse outcomes. The challenges of this extrapolation are directly linked to the limits of existing models (such as DEB models (Jager and Klok, 2010) or Individual Based Models (Beaudouin et al., 2012; Martin et al., 2013)), the choice of their parameters, their complexity and accessibility for AOP developers. In addition to the extrapolation of individual AO to population AO, we discuss the potential to define and measure new AOs directly at the population level, (e.g., demographic characteristics such as density, age-, and/or sex-distributions) and characteristics considering population variation over different time scales (e.g., adaptation, fitness, heritability of traits, etc.). On another level, we discuss the challenges linked to the integration of ecosystemic components. They seem to be the most challenging, notably because of the increased complexity of interactions within and between species, environmental factors and other types of pollution (Knapen, 2021).

After exposing and discussing the main challenges relative to AOP development in the radioecology context, the next section introduces and discusses some existing and emerging approaches to overcome these challenges and build AOP.

2.4.3 Development of AOPs in radioecotoxicology: from molecular integrative approaches to ecosystem modelling

This second section aims to introduce **integrative approaches** useful to determine and link events for AOP building, and to **make some proposals for ecologically relevant adverse outcome pathway building** that overcome several challenges.

First, we refer to approaches that aid in defining relevant molecular events for AOP on radiation. More precisely we focus on the means to use and insert the great amount of “omics” data produced over many years and characterizing modulated biological pathways, in the understanding of adverse outcomes (Brockmeier et al., 2017). We present a focus on tools to screen for sub-individual effects such as omics, DRomics to establish dose-response relationships (Larras et al., 2018) and find the differential radiosensitivity of molecular pathways, helping choosing the most radiosensitive MoA (Mode of Action). Then, an important aspect of AOPs is their potential to quantitatively link key events (Spinu et al., 2020, Conolly et al., 2017). This can help in determining new threshold values for ERA (Paini et al.,

2021). This part discusses current models used in the determination of response-response relationships between KEs and their limits.

Then, to overcome the challenges linked to the integration of more ecosystemic approaches in the AOP framework, some approaches and tools are introduced, notably regarding ecological representativity, i.e., species, life cycle, multi-exposure, population trajectory and interspecies interaction. Specifically, we discuss the potential for cross-species extrapolation between AOPs, using screening tools for conserved pathways and phylogenetic approaches (Doering et al., 2018, LaLone et al., 2016). In addition, to integrate more specific parameters into the AOP, such as sex-specific differences and life stage differences, multidimensional AOPs can be built using tools such as Effectopedia. In line with the challenges linked to exposure to multiple stressors, we discuss the potential for AOP networks to connect AOPs on different stressors and find common pathways (Knapen et al., 2018). Finally, the integration of ecosystemic parameters such as trophic interactions could be integrated into AOPs, notably through mesocosm studies (Roussel et al., 2008, Haanes et al., 2020).

Finally, this review concludes on the potential of AOPs combined with several pre-existing approaches to better understand the effects of radiation stressors on wild species. It highlights the importance of collaborations and sharing of data and taskforces to efficiently fill knowledge gaps, that will perhaps one day enable proper integration of this concept into the ERA framework (Hecker and LaLone, 2019, LaLone et al., 2017).

2.5 CHOICE OF MODEL SPECIES: *CAENORHABDITIS ELEGANS*

2.5.1 Effects of IR on invertebrates

As previously described, species radiosensitivity varies across and within taxonomic groups. Invertebrates, although not the most sensitive, are primordial for ecosystem functioning through the maintenance of nutrient cycling, water storage and primary productivity (Lavelle et al., 1997, Bonzom et al., 2016). Within 2 months after the Chernobyl accident, 90% of soil invertebrate species had perished up to 7km around the power plant (Krivolutskii, 2000). A non-exhaustive meta-analysis of available data shows that even within the invertebrate taxon, species exhibit differences in radiosensitivity (Table 1).

| Species | Individual effect | Acute ED50 (or LOED) (Gy) | Chronic LOEDR (mGy.h ⁻¹)/ Total dose (Gy) – duration of exposure | References |
|---------------------------------|-------------------|---|---|---|
| <i>Folsomia candida</i> | Mortality | 1350 | | (Nakamori et al., 2008) |
| | Reproduction | 21.9 | | (Barescut et al., 2009) |
| | Growth | 144 | | |
| <i>Eisenia foetida</i> | Mortality | 825 | | (Suzuki and Egami, 1983) |
| | Reproduction | 11.1 | <u>Hatching:</u> 4/ 8,7 – 13 weeks | Acute: (Barescut et al., 2009) Chronic: (Hertel-Aas et al., 2007) |
| | Growth | 95 | | (Barescut et al., 2009) |
| <i>Porcellio scaber</i> | Mortality | | >8/ 18.8 – 14 weeks | (Hingston et al., 2005) |
| | Reproduction | | >8/ 18.8 – 14 weeks | |
| <i>Ophryotrocha diadema</i> | Reproduction | | 13.7/ 20.4 – 62 days | (Knowles and Greenwood, 1994) |
| <i>Neanthes arenaceodentata</i> | Mortality | > 500 (LOED) | | (Anderson et al., 1990) |
| | Reproduction | 1-4 (LOED) | <u>Broodsize:</u> 17/ 54 - 132 days (2 gen.) <u>Hatching:</u> 0.19/ 0.55 – 120 days (2 gen.) | Acute: (Anderson et al., 1990, Harrison and Anderson, 1994a) Chronic: (Harrison and Anderson, 1994b) |
| <i>Daphnia magna</i> | Mortality | | >35.4/ 17.8 – 21 days | (Parisot et al., 2015) |
| | Reproduction | | 31/ 11 - 14 days | (Gilbin et al., 2008) |
| | Growth | | >35.4/ 17.8 – 21 days | (Parisot et al., 2015) |
| <i>Caenorhabditis elegans</i> | Mortality | > 1 kGy | | (Hartman et al., 1988) |
| | Reproduction | <u>Broodsize:</u> 30 (LOED) 80 (ED50) <u>Hatching:</u> 40 (LOED) 77 (ED50) | <u>Broodsize:</u> <i>Observed:</i> 42.7/ 2,7 (3 rd gen.) 50/ 3.3 (1 st gen.) 26.8/ 7 (1 st gen. - 11 days) <i>Estimated:</i> | Acute: (Dubois, 2017) Chronic: (Buisset-Goussen et al., 2014) (Lecomte-Pradines et al., 2017) |

| | | | | |
|--|--------|--|---|---------------------------------|
| | | | 18.8/4,9 (1 st gen. - 11 days) <u>Hatching:</u> No observed effect | |
| | Growth | | <i>Estimated:</i> 18.8/4,9 (1 st gen.- 11 days) | (Lecomte-Pradines et al., 2017) |

Table 1 Non exhaustive analysis of literature data regarding effects of acute and chronic exposure to radiation on the main individual parameters of several species of invertebrates. LOED/DR: Lowest Observed Effect Dose/Dose Rate, ED50: Effective Dose observed for 50% of individuals,

As previously highlighted, these data suggest that individuals are more sensitive to chronic exposure scenarios than acute (Dubois et al., 2018) (Pereira et al., 2011).

Among the three main ecologically relevant individual parameters regarding population dynamics, reproduction is the most radiosensitive in invertebrates (Table 1). Moreover, several studies show that threshold values for a decrease in reproduction tend to diminish across generations. For example, in the study of Knowles and Greenwood, reproduction was affected at 13.7 mGy.h⁻¹ on the first generation, but as low as 1.7 mGy.h⁻¹ on the second and third generations (Knowles and Greenwood, 1994). The same was shown for *C. elegans* (Buisset-Goussen et al., 2014, Guédon et al., 2021), *Neanthes arenaceodentata* (Harrison and Anderson, 1994b) and *Daphnia magna* (Parisot et al., 2015).

In addition, one important parameter in the experimental design of these studies is the choice of irradiated life stage (apart from duration exposure). Indeed, depending on the life stage, organisms might exhibit different responses. In adult insects (drosophilae and wasps), DL50 varies between 20 and 3000Gy while embryo DL50 ranges from 1-2Gy (Woodhead, 1998).

Among those model species, *Caenorhabditis elegans* is an androdiecious ubiquitous soil invertebrate, which represents an ecologically relevant model species and could even be used as bioindicators of soil health (Lecomte-Pradines et al., 2014). It has previously been used to characterize radioinduced effects at several biological levels. However, compared to other more ecologically relevant species, the true strength of *C. elegans* is the wide biological knowledge that has been acquired on this organism throughout the years (Sulston and Horvitz, 1977, Consortium, 1998, White et al., 1986). It has provided and continues to provide insights

into fundamental mechanisms of development, biomedical research (e.g., neurobiology, basic physiology, genetics, immunology, cancer biology), species diversification and genome evolution (Wilson-Sanders, 2011). Therefore, *C. elegans* is perfectly fit for our purpose of characterizing molecular determinants of radioinduced reprotoxicity.

2.5.2 Life cycle

C. elegans has a short life cycle (2.5-3 days) consisting of several larval stages as shown in Figure 5. The timing of development is highly dependent on temperature and optimized between 18-22 °C as lower temperatures will slow down its development and egg-laying rate, while higher temperatures will induce stress resulting in the reduction of total broodsize and spawning of males in the population (Byerly et al., 1976, Morran et al., 2009, Anderson et al., 2010). In the present experiments, the optimal temperature used was 19°C. The Bristol N2 strain was used as a 'wildtype'.

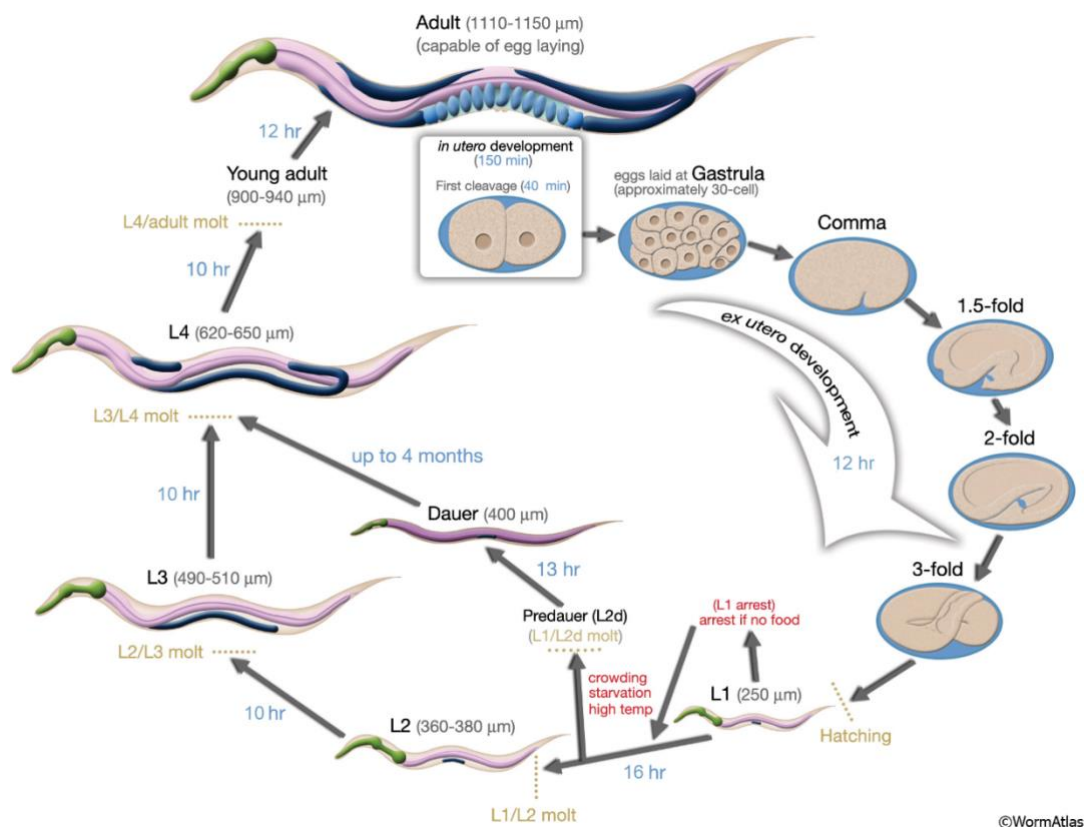


Figure 5 *Caenorhabditis elegans* life cycle at 19°C (adapted from the WormAtlas)

C. elegans life cycle consists of four larval stages (L1-L4) separated by moulting until it reaches the L4/adult molt (Altun et al., 2006). Embryogenesis is the first phase of development

starting from egg fertilization until egg hatching. Embryos start to develop *in utero* for ~150min until they are laid. *Ex utero* development is about 12h, then after hatching, the larvae carry on their development from L1 to adult stage and each larval stage lasts about 10-16h. During embryogenesis, cells undergo a phase of intense proliferation followed by organogenesis/morphogenesis. After hatching, post-embryonic development is triggered upon feeding from bacteria, unless L1 larvae are placed under starvation conditions, in which case they arrest development for up to 10 days (Johnson et al., 1984). During L1 stage, precursor cells for each of the nervous, coelomocyte and reproduction systems (somatic gonad and germline) start to divide (Kimble and Hirsh, 1979). In particular, the development of the gonad, i.e., gonadogenesis, which starts at approximately 7 hours after hatching, is completed in the L4 stage (see 2.5.3). During L2, L3 and L4 stages, cells continue to undergo divisions and to differentiate to form specific tissues and organs until they reach adulthood. Throughout *C. elegans* development, 131 out of the 1090 somatic cells are eliminated via programmed cell death, also called apoptosis (Driscoll, 1995). This phenomenon is referred to as “developmental cell death” and contrasts with stress-induced apoptosis (see 2.5.4). Once its development is completed (adult stage), somatic cells do not enter further mitotic divisions and cell proliferation is restricted to the germline.

In addition, nematodes have the particularity to enter an alternative larval stage if they are faced with external stress factors (e.g., starvation, temperature, population density), called the Dauer stage. This stage can be entered from L1 stage and exited to go straight to L4 stage once their environment becomes favourable again (Wolkow and Hall, 2015).

2.5.3 Reproductive system

Worms start egg fertilization during the L4/YA stage, and egg-laying during their adulthood. Self-fertilized *C. elegans* can produce up to 300 offspring, which can be enhanced by outcrossing with males (up to 1200-1400) (Anderson et al., 2010). Egg-laying lasts about four to six days with a peak between the second and third day after the beginning of egg-laying (Byerly et al., 1976).

2.5.3.1 Germline development

The gonad of *C. elegans* consists of both the germline where gametes are formed, and the somatic gonad, acting as a support for germline development and reproduction (vulva for

fertilization and ovulation, distal tip cell (DTC) signalling germ stem cells (GSC) differentiation, gonadal sheath cells facilitating ovulation, etc.). Each hermaphrodite has two gonad arms joining at the vulva where eggs are laid as shown in Figure 6.

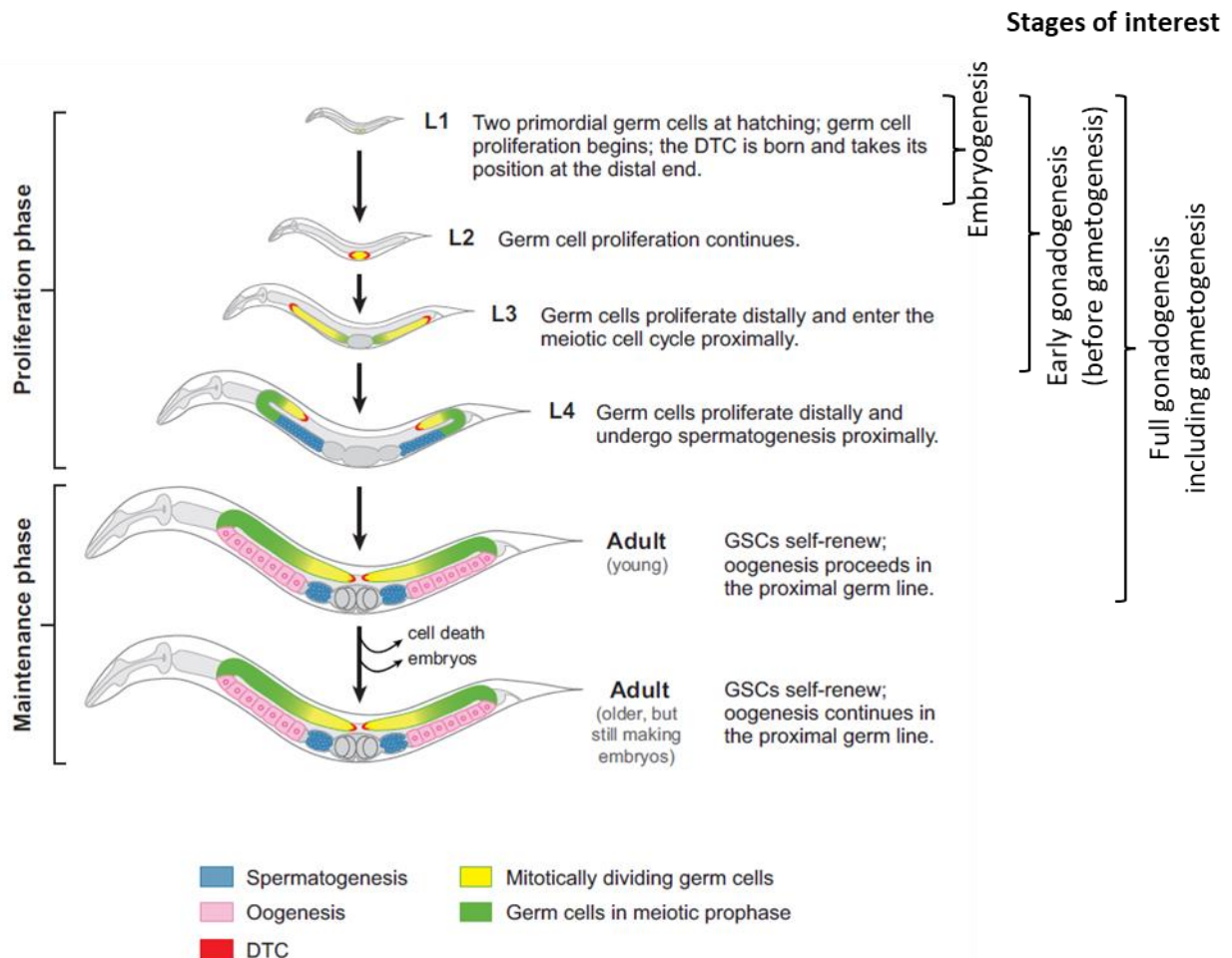


Figure 6 *C. elegans* hermaphrodite germline development (adapted from (Kimble and Crittenden, 2007)). Hermaphrodite germline development takes place in two gonadal arms, which are shown here within the animal as it develops through the larval stages (L1–L4) and into adulthood.

C. elegans are protandrous hermaphrodites meaning they will produce male and female gametes sequentially during their development. A fixed number of spermatozoa (~300) is first produced during the late L3/L4 stage and stored in the spermatheca at the proximal end of the gonad until they can fertilize oocytes. Oocytes are then produced continuously from the beginning of L4/YA stage throughout adulthood.

The development of *C. elegans* germline is spatially and temporally linear which makes it a great tool to easily identify stages of the cell cycle (Figure 7) (Pazdernik and Schedl, 2013).

Germ cell development starts at the L1 stage from the distal end of the gonad where the distal tip cell (DTC), which is a somatic cell, acts to control the mitotic proliferation of GSC (Figure 6) (Kimble and White, 1981). From L1 to late L3 stage, GSC proliferate in the mitotic zone and then enter meiosis at the end of L3 stage to differentiate as spermatozoa. The transition zone between mitosis and meiosis can easily be identified from the crescent-shaped cell nuclei (Figure 7). GC then undergo meiosis I along the germline until they reach the proximal end where they will undergo meiosis II (meiotic maturation of oocytes or spermatozoa). At the end of spermatogenesis at the end of the L4 stage, gamete differentiation switches to oogenesis (Figure 6). The transition from spermatogenesis to oogenesis is part of the sex determination pathway, which is tightly controlled by several factors either autonomous or external (Kimble and Crittenden, 2007).

Based on this understanding of germline development and gonadogenesis, three stages of interest, defined in Figure 6, were identified regarding reproduction outcomes.

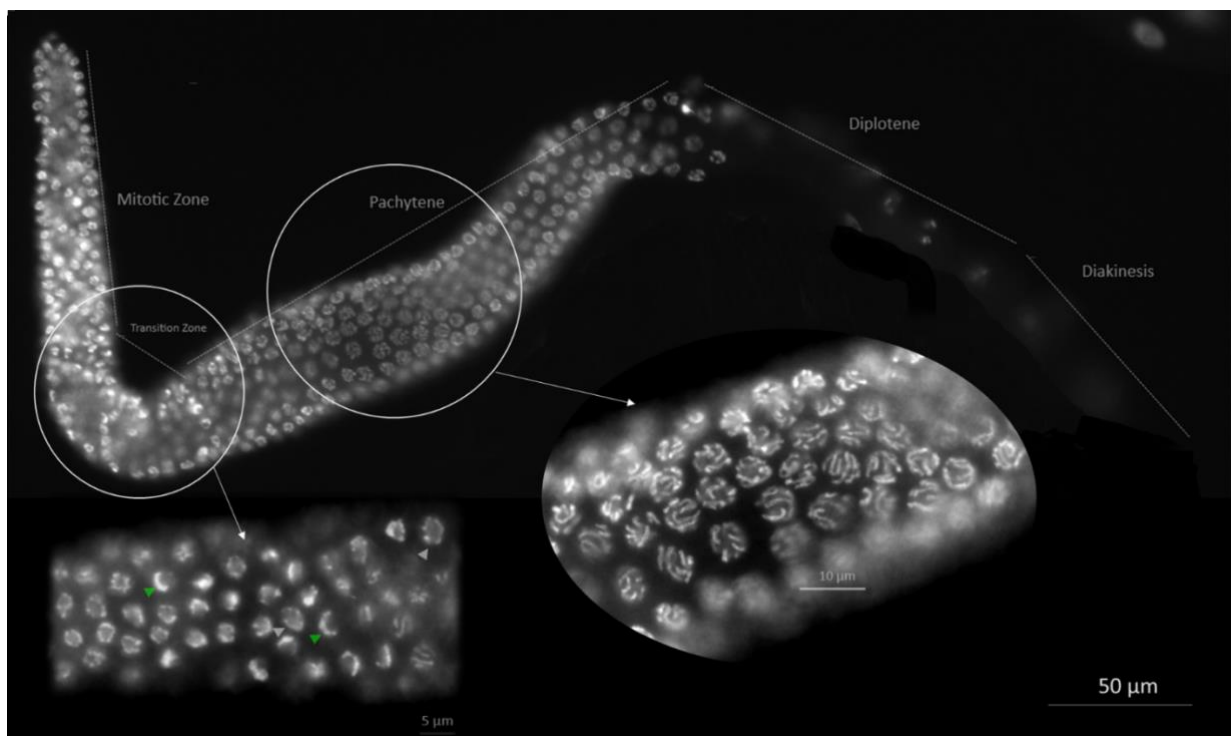


Figure 7 Spatial and temporal development of the germline in hermaphrodite *C. elegans* (DAPI stained, green arrows: crescent shaped nuclei in the transition zone) (original image).

2.5.3.2 Sex determination and gametogenesis

Hermaphrodite *C. elegans* have a female soma and a hermaphrodite germ line producing both male and female gametes. Most differences between the sexes develop post-embryonically by different cell-specific patterns of gene expression. Figure 8 shows the main regulators of the sex determination pathway. In somatic cells, the sex-specific fate of different cells is controlled by the master regulator *tra-1* which acts as a transcriptional repressor in hermaphrodites, promoting female fate (Mathies et al., 2004, Guo et al., 2013, Zarkower, 2006, Ellis and Schedl, 2007). In the germ line, regulatory interactions of additional genes are required to specify sexual fate, i.e., gamete sex determination (Herman, 2005).

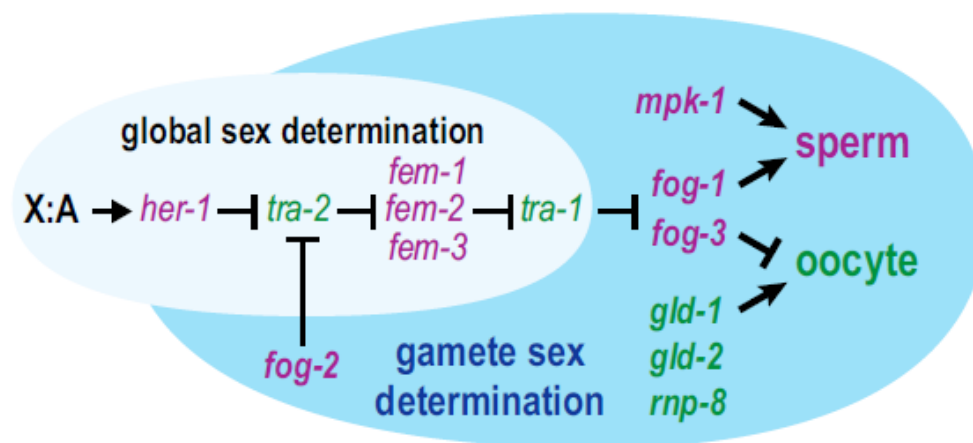


Figure 8 Sex determination module in *C. elegans* (Morgan et al., 2013)

During *C. elegans* hermaphrodite germline development, the transition from spermatogenesis to oogenesis is part of the sex determination pathway in which seven core genes (including *tra-1*) act to control the fate of germ cells (Ellis and Schedl, 2007). The number of spermatozoa is tightly regulated by these genes as overexpression of male-fate promoting genes will induce a higher number of spermatozoa (Yoon et al., 2017a) and mutations in any of these genes even prevents the production of spermatozoa causing germ cells to differentiate as oocytes (Ellis and Kimble, 1995, Hodgkin, 1980). The mechanisms of regulation of this spermatogenesis/oogenesis switch are still unclear. Notably, this switch is regulated by the same genes signalling the control of proliferation and entry into meiosis, suggesting that these two events are interconnected (Kimble and Crittenden, 2007). However, recent findings

overthrew this paradigm and showed that despite having the same regulators, they are two separable events in the germline (Morgan et al., 2013).

Very few studies question whether the sex determination pathway in *C. elegans* can be regulated via environmental cues (Bayer et al., 2020). However, environmental regulation of sex determination is conserved across several taxonomic groups (reptiles, insects, fishes)(Hake and O'Connor, 2008, Devlin and Nagahama, 2002) and several studies point out a change in sex ratio in populations exposed to diverse sources of stress (Cutter et al., 2003, Liu et al., 2021b, Geffroy and Wedekind, 2020).

2.5.4 Main signaling pathways involved in stress response

As previously described, *C. elegans* is one of the most well studied biological systems because of the remarkable simplicity of this model (short life cycle, transparent, 959 post-mitotic cells), for which complete cell lineage (Sulston and Horvitz, 1977), neuronal networks (White et al., 1986) and genome sequence (Consortium, 1998) were established. The major signaling systems have been conserved to a remarkable extent in all animals (Greenwald, 2005a). In particular, *C. elegans* stress response pathways (e.g., in response to oxidative, osmotic, heat stress or hypoxia) have been extensively studied to understand the genetic machineries that underlie a number of human stress-related diseases such as cancer or neurodegenerative diseases (Rodriguez et al., 2013). This part briefly introduces some of the main signaling pathways involved in stress response, especially oxidative stress, and their physiological consequences on the organism.

- Insulin/Insulin-like Signaling (IIS) pathway and downstream targets

Most notable is the insulin/insulin-like growth factor (IGF) signaling pathway (IIS), which involves the forkhead transcription factor DAF-16, a key regulator of lifespan changes in response to environmental and gonadal stimuli (Kenyon, 2010). This fundamental pathway connects nutrient levels to metabolism, growth, development, longevity, and behavior. It is regulated by insulin-like peptide ligands that bind to DAF-2 ortholog of the insulin/IGF-1 transmembrane receptor (IGFR) (Murphy and Hu, 2013). Downstream targets of DAF-2 are for example the transcriptional factors SKN-1/Nrf and FOXO/DAF-16 that regulate various biological processes, such as oxidative stress response (Ogg et al., 1997, Tullet et al., 2017, Shao et al., 2019, Honda and Honda, 1999, HONDA and HONDA, 2002, Oh et al., 2006, Laurent

et al., 2008). IIS pathway acts in somatic tissues, especially the intestine by regulating lipid metabolism, but is tightly linked to gonadal stimuli and stress response in the germline (Ezcurra et al., 2018). Notably, it is involved in the regulation of cell cycle arrest (Baugh and Sternberg, 2006, Fukuyama et al., 2006), proliferation and apoptosis of germ cells (Pinkston-Gosse and Kenyon, 2007, Michaelson et al., 2010), notably through the Ras/ERK signaling pathway (see below) (Burton et al., 2017).

The transcription factor SKN-1 (ortholog of Nrf/CNC proteins in mammals) is known as a regulator of antioxidant and xenobiotic defense but is also implicated in numerous functions of proteostasis and metabolic regulation (Figure 9)(Blackwell et al., 2015). It is activated by stress signals and mediated pathways including the IIS pathway as previously described. Additionally, it controls genes involved in a variety of biological processes such as stress response, lipid metabolism, detoxification, lifespan, etc. (An and Blackwell, 2003, Yoon et al., 2017c, Hu et al., 2017, Tullet et al., 2017). Interestingly, it has been shown to regulate lipid metabolism and lifespan in response to germ cell absence (Steinbaugh et al., 2015, Lemieux and Ashrafi, 2015). Figure 9 illustrates the complexity of SKN-1 functions.

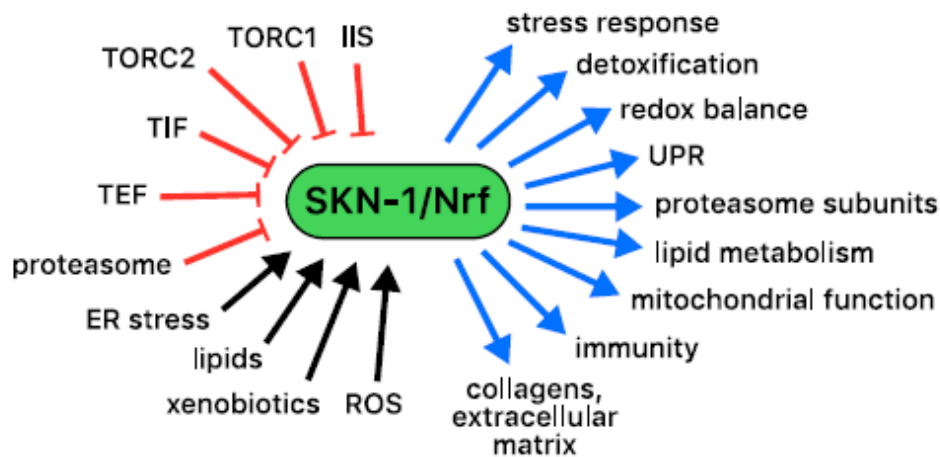


Figure 9 “Pleiotropy of SKN-1 functions. SKN-1 directly or indirectly controls genes involved in a wide variety of biological processes (blue arrows), overlapping subsets of which may be up-regulated by different stresses. SKN-1 is activated by stress signals (black arrows) and senses the activity of multiple cellular processes, some of which are shown here in red. TEF and TIF refer to translation elongation and initiation, disruption of which appears to activate SKN-1 through different mechanisms.” From (Blackwell et al., 2015).

Similarly to SKN-1/Nrf, the FOXO/DAF-16 transcription factor acts to regulate several pathways including lifespan, fat storage, dauer entry and stress resistance (Tissenbaum,

2018). For example, in response to oxidative stress, *C. elegans* has shown an increase of fat storage via DAF-16 activity (Wang et al., 2018). Besides, in several species, loss of germ cells activate this transcription factor in somatic tissues, inducing a lifespan increase (Kenyon, 2010, Hsin and Kenyon, 1999). Specifically, the germline and the somatic reproductive tissues exert counterbalancing influences on lifespan, with the germline preventing, and the somatic gonad promoting lifespan extension. Although precise mechanisms of DAF-16 pathways are still unknown, they allow a better understanding of the connection between lifespan, reproduction and lipid metabolism (see 2.5.5.2).

- Ras/ERK (MPK-1) pathway

Mitogen Activated Protein Kinase (MAPK) are activated upon external stimuli, such as oxidative stress, and contribute to numerous cellular pathways (Gehart et al., 2010). Several types of MAPK contribute to different pathways, among which the extracellular signal-regulated kinases, ERK1/2 in mammals which contribute to cell cycle entry and proliferation (Meloche and Pouyssegur, 2007, Mebratu and Tesfaigzi, 2009). In *C. elegans* the Ras/ERK (MPK-1) pathway is involved in several developmental processes in the germline, including meiotic pachytene progression, pachytene cellular organization, germ cell sexual fate, oocyte growth, differentiation and maturation, and mitosis to meiosis transition as shown in Figure 10 (Lee et al., 2007) (Yoon et al., 2017b, Miller, 2001). In response to external factors, inappropriate ERK signaling contributes to several pathological traits (e.g., over proliferation of cells leading to tumor growth) (Dent et al., 2003, Zhou et al., 2019, Guo et al., 2020). In *C. elegans*, it regulates cell proliferation and DNA damage-induced apoptosis in the germline (Roux and Blenis, 2004, Dent et al., 2003, Rutkowski et al., 2011, Okuyama et al., 2010). Interestingly, this pathway interferes with the IIS pathway in several ways. Notably, MPK-1 phosphorylates SKN-1 on the key sites that are required for SKN-1 nuclear accumulation and thus contributes to the regulation of longevity (Okuyama et al., 2010). Additionally, in the presence of food, DAF-2 functions in the pachytene zone to activate the RAS-MPK-1 pathway and drive meiotic progression (Lopez et al., 2013). These studies once again exhibit a direct link between intestine and germline signalling in stress response.

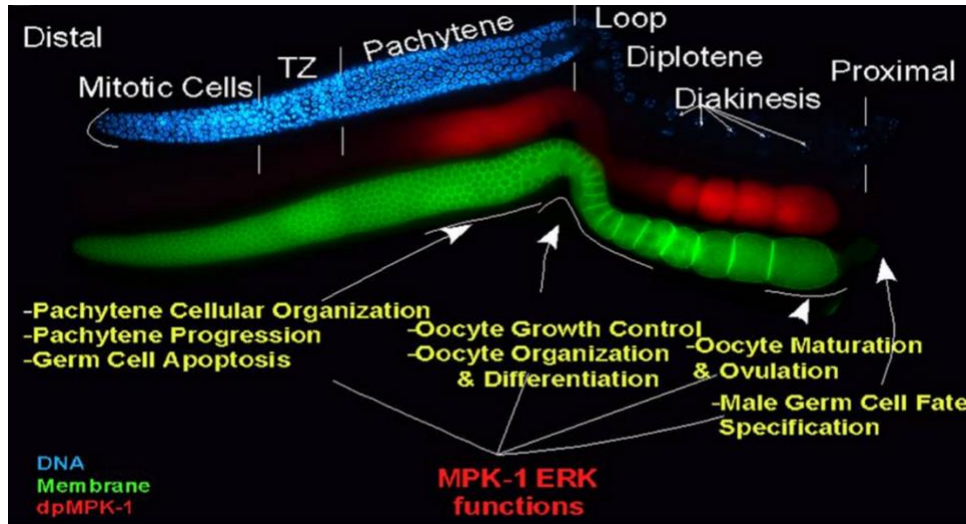


Figure 10 MPK-1 expression zones in the germline and developmental processes associated (Arur et al., 2009).

- GLP-1/Notch signalling pathway

The Notch pathway regulates cell proliferation, cell fate, differentiation, and cell death in all metazoans (Kopan, 2012). *C. elegans* has two Notch proteins encoded by the *glp-1* and *lin-12* genes (Greenwald, 2005b). They contribute to cell fate decisions (e.g., for *lin-12* - vulval cell fate, early gonadogenesis and for *glp-1* – continued germline proliferation, preventing entry into meiosis and gamete formation). The two genes are functionally redundant in some cell fate decisions during embryogenesis. In response to stress, ROS-associated Notch inhibition seems to mediate tumor formation and growth (Packer et al., 2020). In *C. elegans glp-1(lf)* sterile mutants are often more resistant to environmental stressors than fertile nematodes (Wu et al., 2015) (TeKippe and Aballay, 2010). In particular, GLP-1 was found to act upstream of DAF-16 in the IIS pathway (TeKippe and Aballay, 2010, Liu et al., 2021a), once again exhibiting the communication between germline and intestinal signals in the regulation of toxicity of environmental stresses in nematodes.

- DNA damage-induced apoptosis

A major consequence of stress on cell fate is apoptosis. Germ cells in adult nematodes undergo mitotic and meiotic cell divisions, whereas somatic tissues are entirely post-mitotic. Consequently, DNA damage checkpoints in proliferating cells function specifically in the germline, leading to cell cycle arrest and apoptosis, whereas somatic tissues in adult *C. elegans* are more resistant to stress (Ermolaeva et al., 2013, Gartner et al., 2000). DNA damage-

induced apoptosis in the germline is triggered by other regulators than the ones for physiological apoptosis (i.e., normal apoptosis occurring during development and oogenesis) as shown in Figure 11 (Bailly and Gartner, 2013). In both cases and in all cell types in *C. elegans*, the downstream core apoptotic pathway remains the same and its final caspase protein is CED-3 (Malin and Shaham, 2015). In germ cells, apoptosis is restricted to late pachytene stage germ cells at the L4 stage (Bailly and Gartner, 2013). In male germ cells, neither DNA-damage induced nor physiological apoptosis occurs (Bailly and Gartner, 2013, Gartner et al., 2000, Gumienny et al., 1999). As previously described, MPK-1 is involved in physiological apoptosis, as well as DNA-damage induced apoptosis.

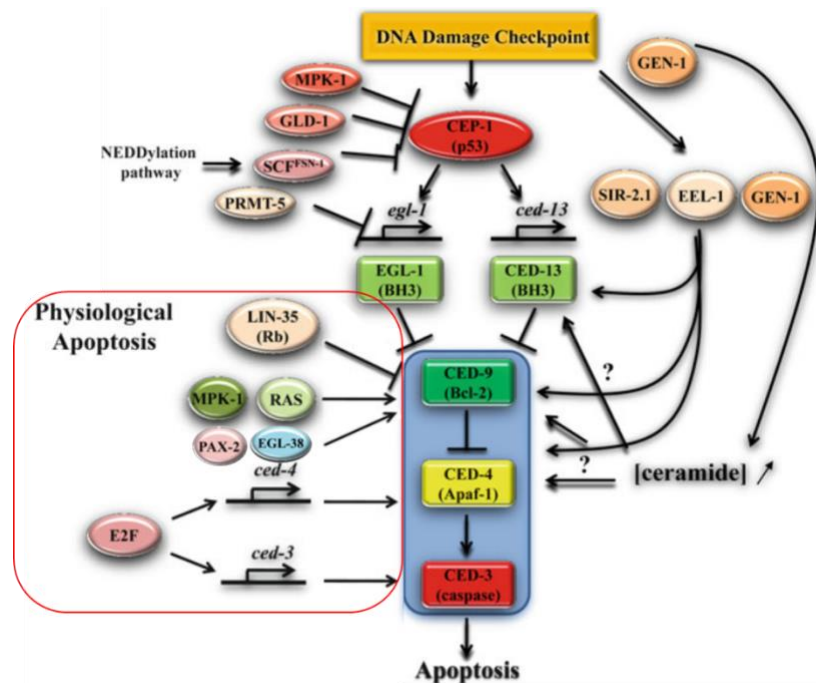


Figure 11 Representation of diverse pathways inducing physiological apoptosis and DNA-damage apoptosis in *C. elegans* germ cells (adapted from (Bailly and Gartner, 2013)). Blue rectangle shows the core apoptotic pathway, common to both DNA-damage induced apoptosis and physiological apoptosis. Red frame shows the known pathways inducing physiological apoptosis.

Although the mechanisms of stress response remain complex and not completely elucidated, the wide knowledge available combined with the potential to use *C. elegans* as a tool for screening and functionally validating these pathways (mutants, RNAi, etc.) is a promising and valuable way to further explore the molecular responses to environmental toxicants.

2.5.5 Effects of ionizing radiation on *C. elegans*

2.5.5.1 Reprotoxic effects

The first studies exploring the effects of IR on *C. elegans* reproduction focused on molecular effects after high-dose acute exposure (DNA damage, apoptosis, etc.) (Sakashita et al., 2010, Bailly and Gartner, 2011). They used *C. elegans* as a tool to explore the molecular mechanisms induced by radiations and had thus no ecological relevance either in terms of dose or exposure duration. The first known study to focus on effects after chronic exposure showed a broodsize decrease after exposure throughout whole development (egg to L4/Young adult stage) as soon as 42.7 mGy.h⁻¹ as well as a decrease in sperm number, increase in cell cycle arrest and increase of *egl-1* expression (involved in radio-induced apoptosis) (Buisset-Goussen et al., 2014). Since then, more studies have focused on the sub-individual effects after chronic exposure to IR. Dubois et al. showed that responses of the proteome differ between acute and chronic exposure (Dubois et al., 2018) and that several proteins modulated after chronic exposure were linked to reproduction processes (Dubois et al., 2019). These studies made it possible to decipher the molecular determinants of radio-induced reproduction decrease in *C. elegans*, but still, some mechanisms remain unknown, such as the cause of sperm decrease, specific effects on oocytes and the timing of occurrence of these responses considering larval development.

2.5.5.2 Effects on lipids

As previously described, IR can damage lipids which can have several physiological consequences on the organism (alteration of lipid metabolism, integrity of cell membranes, etc.). In *C. elegans*, Kuzmic et al. showed an increase of lipid catabolism after irradiation throughout whole development at a total dose of 6.8 Gy (100 mGy.h⁻¹ from egg stage to L4/YA) in N2 strain (Kuzmic et al., 2019). In the proteomic analysis of Dubois et al., vitellogenins were overexpressed after chronic irradiation throughout whole development, suggesting a perturbation of lipid metabolism (Dubois et al., 2019). These proteins are lipid transporters essential for oocyte maturation and embryo development (Perez and Lehner, 2019). Interestingly, these proteins are highly expressed in the *glp-1* sterile mutant which showed an increase in lipid catabolism after chronic exposure to IR as low as 0.5 Gy (7 mGy.h⁻¹ from egg to L4/YA) (Kuzmic et al., 2019). In *C. elegans* as in many species, lipid metabolism and

reproduction are interconnected as shown in Figure 12 (Lemieux and Ashrafi, 2016, Amrit et al., 2016, Ratnappan et al., 2014, Hansen et al., 2013, Wang et al., 2008). Thus, each lipid class might have some dedicated roles, signaling, and regulations in various reproduction processes. However, current studies on the effects of IR on lipids have mainly focused on the regulation of total lipid content (Gomes et al., 2018, Song et al., 2020, Kuzmic et al., 2019) although the understanding of compositional changes in lipid classes and fatty acids should give better information on the mechanisms occurring after a stress (Shmookler Reis et al., 2011, Hulbert, 2011).

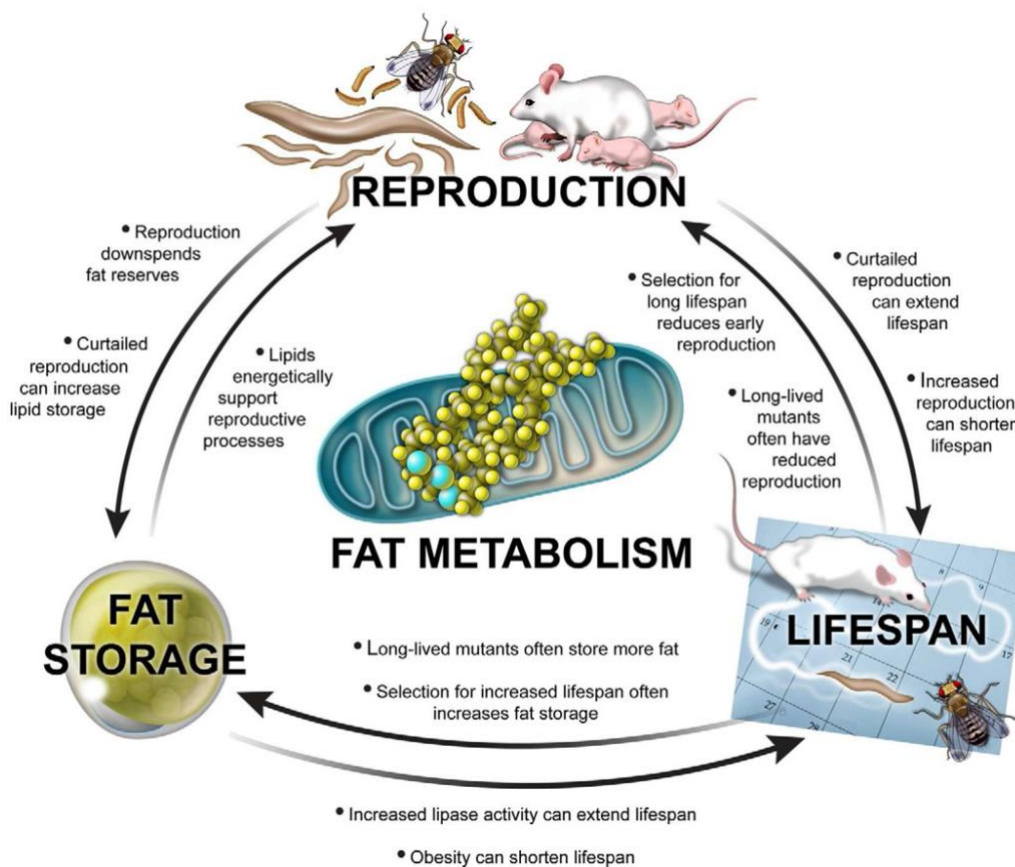


Figure 12 “Reproduction, Fat Metabolism, and Lifespan are Intimately Interconnected. Although the mechanistic cause-and-effect relationships are not yet clear, multiple lines of experimental evidence point to tight links between reproduction, fat metabolism, and aging (see bullet points in figure). For example, observations in worms, insects, and rodents indicate that reproduction (top center) can directly affect fat storage and lifespan. Moreover, increased lifespan (bottom right) is often negatively correlated with reproduction and positively correlated with increased fat storage, whereas fat metabolism (bottom left) influences the energetic cost of reproduction and may directly modulate lifespan.” From (Hansen et al., 2013).

2.5.5.3 Life-stage and cell radiosensitivity

As previously described, radiosensitivity of species depends on the exposed life stage. Precocious organisms are usually more radiosensitive than adults because cells are rapidly dividing during tissue development and thus more prone to damages (Adam-Guillermin et al., 2018). In *C. elegans*, embryos to L1 larvae are more radiosensitive than older nematodes after acute exposure to IR (Hartman and Herman, 1982, Klass, 1977).

At the cellular level, it has been shown that cell radiosensitivity depends on their phase in the cell cycle (Haber and Rothstein, 1969). Cells are least radiosensitive in S phase, then G1 then G2, and most radiosensitive during mitosis as shown in Figure 13. Germ cells that are still differentiating are particularly radiosensitive, and because of their active division, they show irradiation damage very quickly (Tilton and Brower, 1983) (Salam et al., 1970). In contrast, stem cells in G0 phase (arrested phase of the cell cycle) are more radioresistant than stem cells progressing through the cell cycle. This would be because radio-induced lesions on cells in G0 phase are repaired, while in differentiating cells, these lesions can trigger cell death by apoptosis.

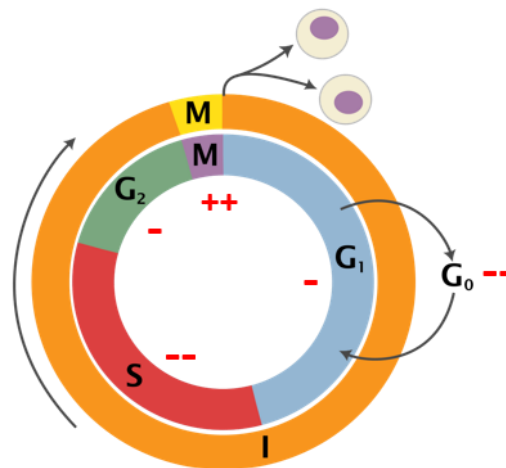


Figure 13 Cell radiosensitivity according to cell cycle phase (++ more radiosensitive, -- less radiosensitive) (adapted from Wikipedia).

Indeed, studies on mammals show a greater radiosensitivity of germ cells at early stages of development (Vergouwen et al., 1995, Lambrot et al., 2007). In the Medaka fish, *Oryzias latipes*, early-differentiating spermatogonia are more sensitive than spermatogonial stem cells (Kuwahara et al., 2002). In *C. elegans*, germ cells undergoing meiosis at the pachytene stage are more radioresistant than embryonic cells (Takanami et al., 2000). These

observations question whether *C. elegans*' spermatozoa are more radiosensitive when they are in the process of differentiation (late L3 stage) than when they are already differentiated (from L4 stage).

Previous studies on multigenerational exposure showed that the second generation irradiated exhibited increased effects compared to the parental irradiation (Buisset-Goussen et al., 2014). In their experimental design, the parental generation was not irradiated from the beginning of embryogenesis, while the second generation was exposed *in utero*. This could mean that early embryogenesis is particularly sensitive to irradiations.

C. elegans embryonic development can be divided into two phases. An "early" phase marked by a rapid division of the cells in mitosis (550 cells in 6h), and a "late" phase of 8h during which the cells do not divide any more. During this late phase, the germ cell precursors are arrested in the G2 phase, during which homologous recombination of chromosomes takes place. This germ cell functionality has the evolutionary interest of facilitating chromosome homologous recombination in order to protect the genetic information transmitted to future generations. Indeed, germ cell radioresistance appears to rely exclusively on homologous recombination (Lemmens and Tijsterman, 2011, Takanami et al., 2003). Therefore, because cells are rapidly dividing in early embryonic development and might thus be more radiosensitive, it seems important to consider this life stage in future irradiation designs.

3 RESEARCH AXES AND METHODOLOGY

3.1 RESEARCH AXES

The main objective of this project was to better understand the mechanisms underlying the radioinduced reprotoxic effects in the nematode *C. elegans*. Two research axes were then defined. The first axis aimed to characterize these mechanisms via 3 main research questions, as follows:

- 1) Is there a more radiosensitive developmental stage regarding reproduction outcome?
- 2) What is the connection between lipids and reproduction in response to IR?
- 3) Which developmental pathways in the germline are involved in response to IR?

The second axis was to better assess the links between the identified effects and mechanisms and structure them into an AOP scheme. This enabled assessment of their relevance to improve environmental risk assessment.

3.1.1 Axis 1: Characterization of mechanisms underlying radioinduced reprotoxic effects

3.1.1.1 Question 1: *Is there a more radiosensitive developmental stage regarding reproductive outcome?*

Considering the previous data acquired on radioinduced reprotoxicity and the developmental steps of *C. elegans*, identifying critical stages of development regarding reprotoxic response would enable better characterization of the mechanisms and their timing of occurrence. Previous results showed that reprotoxic effects were increased in the second generation (F1) irradiated from *in utero* in the parental generation, compared to parental irradiation only (F0) irradiated from egg laid stage. Thus, we postulated that *in utero* irradiation of eggs might be accountable for the increased observed effects. Additionally, we considered different steps of *C. elegans* germline development to be potentially critical targets of IR. Thus, three developmental stages were identified:

- Hypothesis 1: The embryonic stage would be the most radiosensitive because damages to **primordial somatic and germ cells** would affect the development of the gonad (i.e., early embryogenesis from *in utero* to mid L1).
- Hypothesis 2: The most radiosensitive stages with respect to reproduction would be the stages of **rapid germ cell proliferation** (early development of the gonad, i.e., from embryo to late L3 stage).
- Hypothesis 3: The stage during which meiosis takes place (i.e., from late L3 to L4/YA) would be the most radiosensitive because **gametes are more sensitive during their formation than when differentiated**. This hypothesis was considered through the common scenario of exposure of previous studies, i.e., irradiation from egg stage to L4/YA, adding the *in utero* exposure. This exposure scenario is basically the same as the one in hypothesis 2 but also includes gametogenesis; thus, the comparison between the two scenarios will allow us to understand effects during gametogenesis.

To answer this first question, a study of the reproductive parameters (at the individual and cellular level) was carried out after irradiation until mid-L1, late L3 and L4-YA stages (see experimental design).

3.1.1.2 Question 2: What is the connection between lipids and reproduction in response to ionizing radiation? How do they contribute to the decrease in reproduction?

As previously described, lipids play an important role in signalling reproduction processes and vice versa. Previous studies showed a misregulation of lipid metabolism in the nematode upon chronic exposure to IR, such as an overexpression of vitellogenin. However, no difference in the total lipid content in the N2 irradiated organism was found, while germline-less mutants *glp-1* showed a decrease of neutral lipid content, associated with lipid catabolism. Thus, one objective of this thesis was to investigate more precisely the links with reproduction decrease.

Hypothesis: Previous studies only measured total lipid content which could mask more subtle responses of specific lipid classes. Thus, we hypothesized that lipid metabolism is regulated differentially depending on lipid types (i.e., neutral lipids, polar lipids), and that **not all types of lipids contribute to reproduction decrease equally**.

In order to investigate this hypothesis, we analysed polar and neutral lipid content and 20 fatty acids after chronic exposure to IR (following the life-stage dependent approach) and conducted a detailed analysis of literature to understand their link with reproduction.

3.1.1.3 Question 3: Are developmental pathways in the germline involved in the response to ionizing radiation? How do they contribute to the decrease in reproduction?

Prior theses and other studies showed that several pathways in the germline were involved in the response to exposure to IR. Specifically, several items of evidence showed a defect in proliferation processes (cell cycle arrest, overexpression of the MCM complex involved in DNA replication), increased apoptosis (overexpression of *egl-1*) and decrease in sperm number. However, no causal relationship was established between these stress responses in the germline and effects on gametes. On this basis, we thus screened three potential pathways involved both in stress response (i.e., apoptosis, MAPK pathway) and developmental pathways in the germline, such as the sex determination pathway.

Hypothesis 1: The first pathway of interest identified was the Ras/ERK (MPK-1) pathway. As previously described, this pathway is involved both in stress response (e.g., apoptosis, cell proliferation) and developmental processes in the germline (e.g., sex determination pathway, oocyte maturation and ovulation). Precisely, the hypothesis was that **MPK-1 activation would be increased** upon chronic exposure to IR.

Hypothesis 2: In screening for pathways both involved in stress response and germline development, we identified the TRA-1 protein. This protein is at the crossroad of several pathways that were all involved in stress response (upstream regulator of *egl-1*, regulator of vitellogenesis) and is also a major regulator of the sex determination pathway (including the spermatogenesis/oogenesis switch). Therefore, the hypothesis was that **TRA-1 expression would be modulated in response to chronic IR**, regulating all these pathways and potentially explaining the sperm decrease.

Hypothesis 3: The last pathway assessed was the core apoptotic pathway. Previous studies showed that apoptosis was increased upon chronic exposure to IR. However, it was not known how much it contributes to reproduction decrease. The hypothesis was that **sperm**

decrease is not fully responsible for reproduction decrease, but oocytes also contribute, via apoptosis.

Each of these pathways were investigated using different techniques (mutants, immunostaining, transgene) and roughly followed the same global experimental design.

3.1.2 Axis 2: Data structuring into the AOP framework

As previously described, AOPs have the potential to structure available data to causally link events from molecular scale to individual scale or beyond. In this sense, they can be helpful for highlighting relevant data for environmental risk assessment. Thus, developing an AOP scheme to structure our data was a main objective of this thesis.

The AOP scheme was developed almost in parallel to the first axis and was helpful to identify knowledge gaps and causal links to be assessed between the effects. After the collection of sufficient data, the scheme was developed and discussed regarding its relevance for environmental risk assessment (e.g., potential extrapolation with other species, pollutants, etc.).

3.2 GLOBAL EXPERIMENTAL DESIGN

For each of these questions, the same global experimental design was used. Worms were chronically irradiated throughout different life stages as showed in Figure 14. To also consider possible effects of radiation during early embryogenesis, worms were irradiated *in utero*. Thus, SC1 corresponds to irradiation during early and late embryogenesis (i.e., from *in utero* to mid-L1 stage). To consider effects of radiation prior to gametogenesis, worms were irradiated during SC2, which corresponds to ‘early development’ (i.e., *in utero* to late L3, right before the beginning of spermatogenesis). Finally, to consider effects during whole development, and to fit with previous research projects (Buisset-Goussen et al., 2014, Dubois et al., 2018, Dubois et al., 2019), worms were irradiated following SC3 (i.e., *in utero* to L4/YA stage). At the end of each scenario (except SC3), worms were placed in ‘recovery’ until they reached the L4/YA stage at which the analyses were performed. To assess the effects of irradiated bacterial food on lipid metabolism, another scenario (OP50(i)) was added to the experimental design. Non-irradiated nematodes were treated as controls but grown on

previously irradiated bacterial lawn. The bacteria were irradiated under the same exposure condition as SC3.

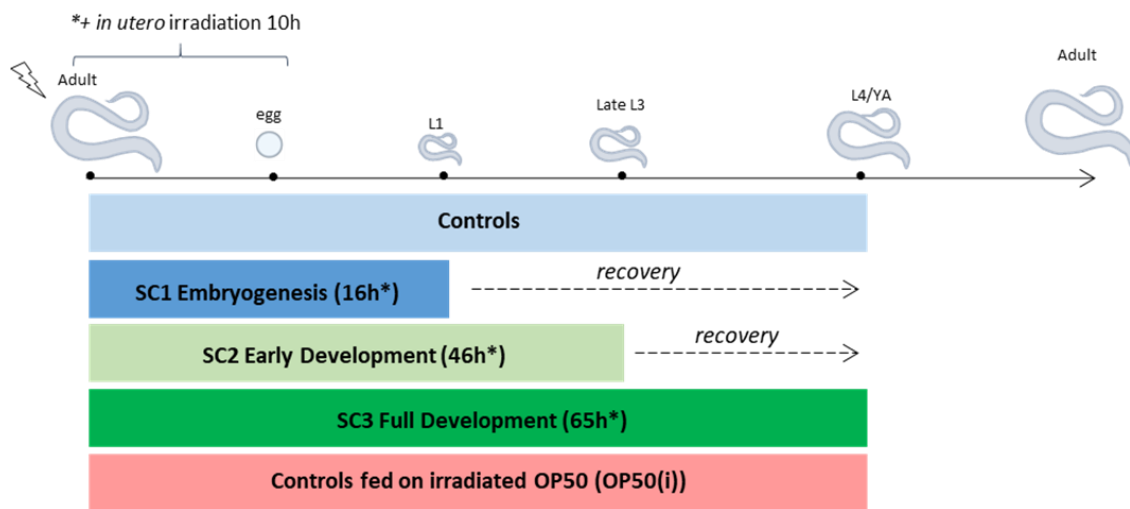


Figure 14 Experimental design for analysis of life-stage dependent reprotoxic effects (SC: scenario, OP50: *E. Coli* strain, L1-L4: *C. elegans* larval stages, YA: Young Adult).

The irradiation facility used was the MIRE platform (Mini Irradiator for Radioecology), in which petri dishes were placed perpendicularly and at the closest distance to the source (Figure 15), which rendered the highest possible dose rate in this facility, i.e., $50 \text{ mGy}\cdot\text{h}^{-1}$.

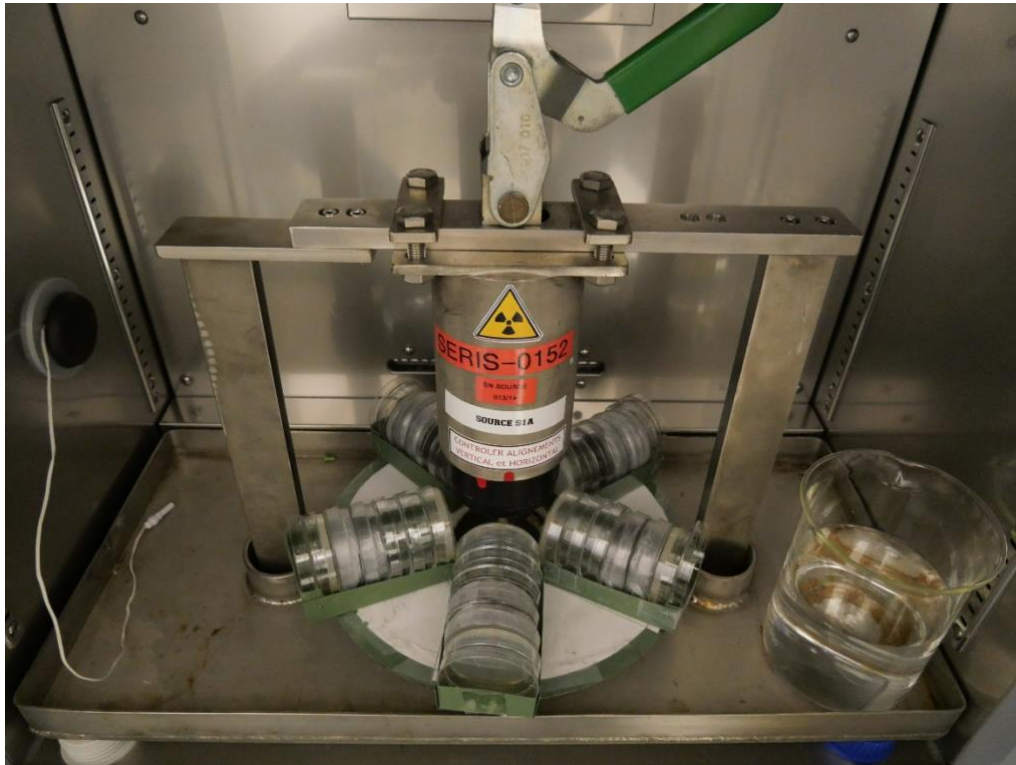


Figure 15 MIRE irradiation device. Petri dishes are placed vertically at the closest distance around the Cs-137 source (for a dose rate of $\sim 50 \text{ mGy}\cdot\text{h}^{-1}$).

4 RESULTS

4.1 AXIS 1: CHARACTERIZATION OF MECHANISMS UNDERLYING RADIOINDUCED REPROTOXIC EFFECTS

In this part, the main results are introduced in scientific articles. For each article, a short synopsis presents the context, main findings, and conclusions. Articles can be found at the end of the manuscript.

4.1.1 Question 1 & 2: “Deciphering Differential Life Stage Radioinduced Reproductive Decline in Caenorhabditis elegans through Lipid Analysis”

[see **Paper I** p. 86]

Synopsis: To better understand the role of lipids in the reprotoxic response to IR, lipid analyses were conducted following the previously described life-stage dependent experimental design. Hence, the following paper responds to both questions of lipid role and life-stage dependent effects. These analyses showed that the regulation of lipid metabolism, just like reprotoxic effects, are life-stage dependent. Effects on broodsize appear as soon as embryogenesis is irradiated, but are more deleterious after exposure throughout whole development, suggesting an accumulation of the induced effects. Lipids follow complex opposite trends depending on irradiated life stages. They seem to be differentially regulated by signals from both the germline and the soma according to the life stage. A close analysis of the literature and previous lipid analyses on sterile mutants enabled us to propose a conceptual model deciphering the complex interaction between lipids and reproduction in response to chronic IR.

4.1.2 Question 3: “Developmental pathways involved in the broodsize decrease in *C. elegans* exposed to ionizing radiation”

[see Paper II p.106]

Synopsis: Several lines of evidence point to the fact that IR might alter development of *C. elegans* germline (e.g., alteration of cell cycle (repression of the MCM complex, cell cycle arrest), overexpression of proapoptotic gene *egl-1*), probably responsible for the observed decrease of sperm number and ovulation rate. However, no direct link between these events was established and the weight of evidence for these effects is still low. This paper aimed to investigate the effects of several essential developmental pathways in the germline, using mutants, transgenes and immunostaining techniques. Precisely the Ras/ERK (MPK-1) pathway, apoptotic pathway and sex determination pathway through TRA-1 were investigated, each having an essential role in *C. elegans* germline development including gametogenesis and stress response. Results showed that several pathways act in parallel to induce a decrease of both oogenesis and spermatogenesis. In particular, radio-induced apoptosis is responsible for ovulation rate decrease, while it does not affect spermatogenesis. Interestingly, these results show that in *C. elegans*, sperm decrease is responsible for about 90% of radio-induced broodsize decrease, while ovulation decrease accounts for only 10%. In parallel to the apoptotic response, IR seems to induce a defect in germ cell proliferation, in line with previous evidence. These findings show that even though *C. elegans* sperm is more radiosensitive than oocytes and contributes more to broodsize decrease, oocytes are still affected by IR. This difference in gamete sensitivity could have broader implications after exposure throughout several generations.

4.2 AXIS 2: DATA SYNTHESIS THROUGH THE AOP FRAMEWORK

Already initiated as soon as experimental results were obtained, this part of the project has been benefiting from a European dynamic via the constitution of a WG of interest during the Melodi/Alliance⁵ AOP Workshop for radiation effects in 2021. Due to its ecological

⁵ MELODI (Multidisciplinary European Low Dose Initiative) <https://melodi-online.eu/> // Alliance: <http://www.er-alliance.org/>

relevance, one working group has been dedicated to the 'AO Reproduction decline' with the 6 European contributors (SCK-CEN, niva, IRSN, CERAD), aiming to find a way to build a consensual AOP valid for a few environmental species, constituting subgroups. Since reproductive strategies may influence the underlying mechanisms of reprotoxicity, the objective of each subgroup is to find a balance between the accuracy of defined terms regarding taxonomic specificities and broad enough terms that can be connected to other taxa. The most common terminology for reprotoxic AO is 'reproduction decrease' but existing AOPs might use different terms according to specificity of measured events, taxa, etc. (e.g., 'cumulative fecundity and spawning', 'fertility impaired', 'decrease of egg production'). The importance of terminology is essential in the AOPwiki database, as when a user chooses a term and proposes their own AOP, the terms will be automatically connected to existing AOPs using the same terminologies, in order to create AOP networks. Therefore, a consensus is needed to standardise terms for reprotoxic AOPs. The *C. elegans* subgroup enabled the discussion and insertion of the relevant results of this thesis in the AOP scheme #396 (CERAD Collaboration). Still in progress, this WG should lead to a short paper presenting several case studies for different taxonomic groups (among which, AOP on nematodes), highlighting their commonalities and discrepancies, and thus make AOPs more comparable and connectable into AOP networks. This was done in parallel to the scheme presented in the following part.

In line with the context of this project, most of the data obtained in the present thesis were structured to fit the AOP framework. The biological domain of applicability of the present AOP concerns hermaphrodite *C. elegans*, at the L4/YA stage but could be extended to the nematode taxa based on their similarities in terms of reproductive biology. In the following paragraphs, all KEs and KERs are detailed and assessed based on the WoE criteria previously described. In line with the current work on the AOP-Reproduction WG, all KEs present in this scheme were carefully chosen considering the balance between their description, frequency of use in the AOPwiki database, and accuracy regarding our own data. The AOP scheme presented in Figure 16, has not been submitted to AOPwiki and is only presented here to summarize the collected data.

Deposition of energy leading to reproduction decrease via gametogenesis impairment (Dufourcq Sekatcheff et al, 2021)

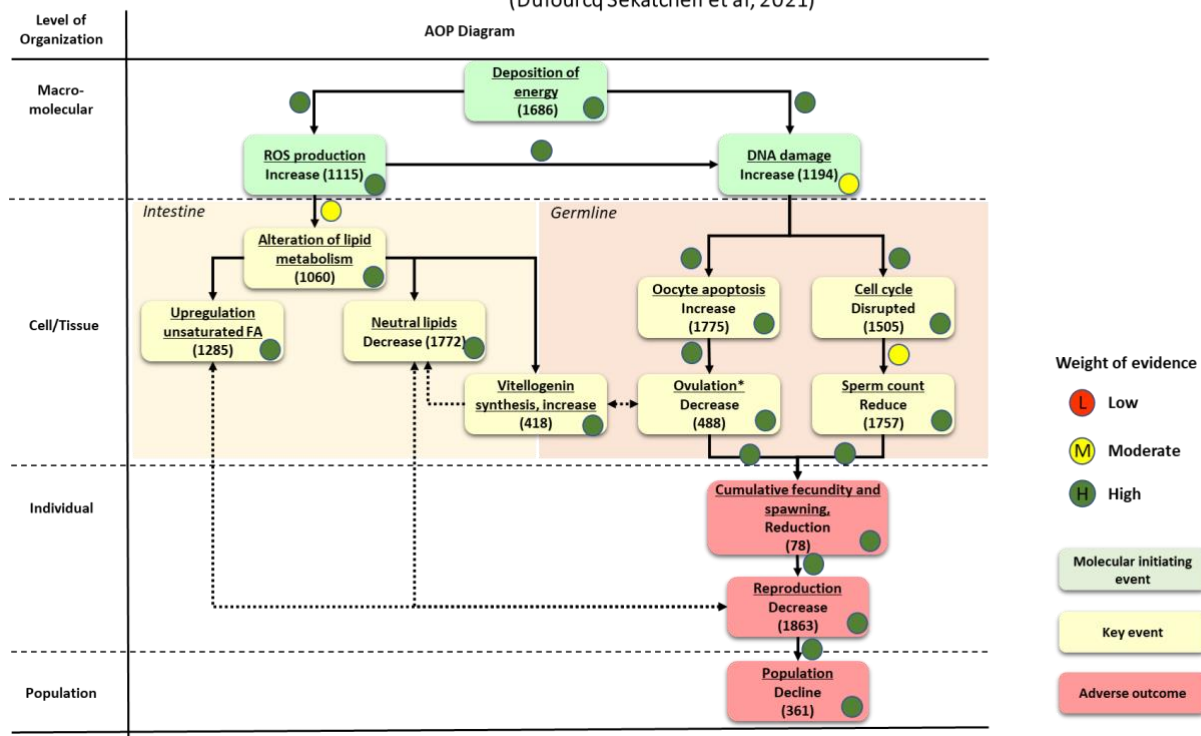


Figure 16 Proposed AOP scheme for deposition of energy leading to reproduction decrease via gametogenesis impairment (Full arrows show direct link between two KEs (i.e., no intermediary KE identified between the two KEs), dotted arrows show indirect link (i.e., several existing KEs between the two KEs but that have not been clearly identified and linked yet)).

- Description and WoE for MIE, KE, AO and KER

Three MIE were identified that are common to most AOPs on ionizing radiation. First, ionizing radiation induces a Deposition of Energy (#1686) on biomolecules, leading to either direct damage of biomolecules, in particular DNA (#1194), or indirect damage (via water radiolysis) caused by the increase in ROS production (#1115). Excessive ROS production can lead to oxidative damage in several biomolecules including lipids and DNA. The choice of MIE regarding ionizing radiation is still a subject of debate within the radiology community. By definition, the MIE should be the first biological event occurring upon interaction with the toxicant. In the case of chemicals, for example, it is the interaction of the toxic molecule with a biological target ligand. In the case of radiation, ionizing radiation induces a deposition of energy within the biological medium, inducing both direct ionization of biomolecules and oxidative stress via water radiolysis, which makes it difficult to isolate a single biological target.

Therefore, in the present scheme, and consistent with previous ionizing radiation AOPs (e.g., AOP#396, AOP#272, AOP#387, AOP#388) these three events were defined as MIEs, pending consensus within the community. ROS production was increased in the whole organism upon chronic exposure to gamma radiation leading to oxidative stress in the germ cells (Maremonti et al., 2020c), leading to a high WoE. For DNA damage (#1194) direct evidence was shown in developing embryos (Maremonti et al., 2019). Specifically in the L4/YA stage, only indirect evidence was found through an increase of cell cycle arrest in the germline (#1055) (Guédon et al., 2021, Buisset-Goussen, 2014), the increased expression of DNA replication (MCM Complex) and DNA repair (*rad-54*, *chk-1*, *hus-1*, *umps-1* and *rpa-2*) genes and proteins (Maremonti et al., 2019, Dubois et al., 2018, Guédon et al., 2021). These elements contribute to a moderate WoE for the assessment of this KE.

An excessive production of ROS in the organism generally leads to oxidative damage of several biomolecules, including lipids, leading to an alteration in lipid metabolism (#1060) in many species (AOP#413, AOP#299, AOP#130, AOP#57). However, in our conditions, the link between ROS production and alteration of lipid metabolism was not directly assessed. In this AOP, alteration of lipid metabolism was described as several effects on lipids. An upregulation of unsaturated fatty acids (#1285) was directly measured, in particular MUFAs (Mono-Unsaturated Fatty Acids) (Dufourcq-Sekatcheff et al., 2021). However, the essentiality of this KE was not assessed regarding reproduction decrease, but some evidence points towards the fact that it could be a consequence of germline defects (Dufourcq-Sekatcheff et al., 2021). The same goes for the decrease of neutral lipids (#1772) which is an important marker of autophagy and was observed from several lines of evidence either directly (quantification of neutral lipids) (Dufourcq-Sekatcheff et al., 2021, Kuzmic et al., 2019), or indirectly (overexpression of *atg-3*, *atg-9*, *ces-2* and *rab-7* genes linked to autophagy) (Maremonti et al., 2019). The link between this KE and reproduction decrease is still unclear, however it could also be a consequence of germline defects (Dufourcq-Sekatcheff et al., 2021). Thus, even though the WoE for these KE is high, the causal link leading to reproduction decrease has not been well established.

Directly at the interface between effects on lipids in the intestine and germline defects, an overexpression of vitellogenins was previously found (Dubois et al., 2019). However, like other

KEs linked to lipid metabolism, the exact link between this KE and reproduction was not established. It is known that vitellogenins are transporters of yolk granules from the intestine to the germline contributing to the maturation of oocytes and development of embryos (Dubois et al., 2019). Thus, their overexpression could either be a consequence of decreased oogenesis (meaning vitellogenins would not be internalized into oocytes and thus accumulate), and/or a consequence of increased yolk granule production in response to lipid misregulation. An increase in PC content (primary component of yolk granules) was found (Dufourcq-Sekatcheff et al., 2021) and supports the latter, but no additional evidence was found that could explain the causal link between vitellogenin increase and reproduction decrease.

In the germline, the identified KEs were essential and causally linkable to reproductive outcomes. This section is partly redundant with the AOP #396 built in collaboration with the NMBU. The first subsequent KEs identified in the germline, i.e., disruption of cell cycle (#1505) and apoptosis in oocytes (#1775) are direct consequences of DNA damage. These two KEs led to parallel pathways of events affecting either spermatocytes or oocytes. Disruption of cell cycle (i.e., cell cycle arrest in our case) was directly observed in the mitotic region of the germline (Guédon et al., 2021, Buisset-Goussen, 2014). This KE is suggested to be a cause of the reduction of sperm count (#1757) which was a phenotype found in several studies (Dufourcq-Sekatcheff et al., 2021, Maremonti et al., 2019, Guédon et al., 2021). Indeed, since there is a fixed number of spermatocytes, a reduction of the pool of germline stem cells due to cell cycle arrest would lead to a decrease in subsequent gametes produced. However, the causal link was not clearly established, and we thus set the KER (Key Event Relationship) to be moderate. In parallel, oogenesis was not affected via this cell cycle arrest, but rather via apoptosis. DNA damage induced apoptosis (which is only occurring in oocytes in *C. elegans* (Jaramillo-Lambert et al., 2010)) for which there is direct evidence from the present manuscript (*ced-3* mutant) and previous evidence of overexpression of radio-induced pro-apoptotic gene *egl-1* (Buisset-Goussen, 2014, Guédon et al., 2021) and in situ visualization of germ cell corpses (Maremonti et al., 2019). This increase in apoptosis was found to be fully responsible (*ced-3* mutant) for the subsequent ovulation rate decrease observed in Dufourcq-Sekatcheff et al. (2021), showing that cell cycle arrest does not affect oocytes.

Finally, the impairment of both oogenesis and spermatogenesis led to our first AO which was the reduction of cumulative fecundity and spawning (#78) described as the total number of offspring produced by the organism (i.e., the broodsize). In order to keep the potential to connect this AOP to other reprotoxic-AOP the subsequent AO 'Reproduction decrease' (#1863) was added, which is a broader term that would fit with other biological domains of applicability.

At the population level, chronic irradiation at the same dose rate for several generations has been shown to induce a decrease of population size linked to a decrease in global population fecundity (Quevarec et al., submitted).

- Knowledge gaps and discussion

Structuring these data into the AOP framework enables us to highlight the knowledge gaps regarding essentiality of KE and KERs. Here, it is clear that the cause of sperm count reduction, especially its link with cell cycle arrest, needs to be further assessed. Interestingly, Maremonti et al. (2019) showed that genes involved in chromosome segregation during spermatid meiosis (*smz-1* and *smz-2*) were down-regulated, suggesting that another cause of sperm decrease could be defective meiosis. Besides, (Guédon et al., 2021) found a decrease in spermatid activation (i.e., the process by which spermatids are fully formed and develop into functional spermatozoa) upon exposure under the same conditions. This result suggests that not only spermatogenesis is affected by IR, but also spermiogenesis, which could contribute to the observed broodsize decrease.

The ability to integrate the data obtained on lipids into the AOP framework is limited as, by definition of OECD guidelines, these KE do not seem to be essential regarding reproductive outcomes but rather a consequence of reproduction decrease. They could also be more easily linked to other outcomes such as longevity or growth, but as reproduction is the most radiosensitive individual parameter, it was not relevant to focus on other outcomes regarding ecological protection criteria.

However, in some AOPs, effects on lipids were successfully linked to reproductive outcome. For example, AOP#299 'ROS production leading to population decline via reduced FAO' showed that a decrease in fatty acid β -oxidation induced oogenesis decrease via a

reduction of mitochondrial ATP synthesis, in *Daphnia magna* upon exposure to ionizing radiation (Song et al., 2020). The authors suggest that the taxonomic applicability could cover a wide range of aquatic species that produce oocytes for reproduction, such as crustaceans, insects and fish. In *C. elegans*, it was indeed found that exposure to IR led to a dose dependent increase in mtDNA copy number, a marker of mitochondrial dysfunction (Maremonti et al., 2020a). However, no clear causal link between mitochondrial dysfunction and reproduction was established. Transcriptomic analysis of *C. elegans* exposed to ionizing radiation revealed that mitochondrial dysfunction is more likely linked to muscular functions, lipid metabolism and general non-targeted responses such as apoptosis, autophagy, nuclear DNA damage, genomic instability (Maremonti et al., 2020c).

Other common KEs are found across taxa and for different types of stressors (e.g., chemicals). For example, effects on vitellogenin uptakes in oocytes were found in several AOPs on several fish species (Doering et al., 2018, Villeneuve, 2018) upon exposure to several types of chemicals, leading to a decrease in cumulative fecundity and spawning. While many aspects of the biology underlying this AOP are largely conserved across oviparous species, its relevance to species other than fish, or to species with different reproductive strategies, has not been established.

Thus, AOPs facilitate access to existing data to better assess the relevance and linkage of identified effects and the potential to expand across taxa.

5 DISCUSSION AND PERSPECTIVES

In the context of Environmental Risk Assessment (ERA) and to improve the characterization of chronic effects of ionizing radiation (IR), this thesis project aimed to decipher the molecular mechanisms leading to radioinduced reprotoxic effects in the nematode *C. elegans*. This objective was approached from two main angles. First, we focused on the characterization of reprotoxic mechanisms through different research questions: life-stage sensitivity, connection between lipids and reproduction and stress response pathways in the germline. Second, the data acquired during this first axis was structured to fit the AOP framework, i.e., assessment of the weight of evidence for each effect and establishment of causal links between effects.

This part discusses the main findings of this project, their interpretation in light of other studies and implications for the ERA context. Some perspectives are suggested within the discussion.

5.1 CHARACTERIZATION OF REPROTOXIC MECHANISMS

5.1.1 Radioinduced reprotoxic effects are life-stage dependent

The question of life-stage sensitivity was of major importance to better understand the mechanisms of reproduction decrease. The majority of current knowledge on the effects of IR on biota is essentially on the adult life stage, although, as previously described, precocious stages are often more radiosensitive (see 2.5.5.3). Thus, identifying the most critical life stage regarding reproductive outcome was of interest for 1) increasing the knowledge of life-stage radiosensitivity and potentially finding new threshold values of reprotoxic effects in response to chronic IR, and 2) narrowing down the possible response pathways involved in reprotoxic effects in respect to their timing of occurrence during the organism's development. We thus focused on three life stages in *C. elegans* that correspond to developmental key steps regarding reproduction (i.e., embryogenesis, early gonadogenesis and whole development).

In doing so, we found that reprotoxic effects are indeed life-stage dependent. Irradiation of early life stages, i.e., embryogenesis and early gonadogenesis, showed a decrease of 11 and 12% respectively in the total broodsize, with no effect on sperm decrease. Irradiation

throughout the whole development (as previously performed), was found to be the most critical scenario regarding reprotoxic outcomes with a decrease of total broodsize of ~35% associated with a reduction of sperm count of ~23% and a decrease of egg-laying rate throughout the reproductive period.

Previous studies on nematodes had shown an increased sensitivity of the second generation (irradiated as early as *in utero*) compared to the first generation (irradiated from the egg laid stage) (Buisset-Goussen et al., 2014). We suggested this difference was due to exposure during very early embryogenesis, so we set the irradiation start *in utero*, by irradiating the parental generation during egg formation of progenies. This very early damage could probably explain the 11 and 12% decrease that we observe upon exposure of early stages, which was not observed in different experimental settings (e.g., starting irradiation in L1 stage or egg stage) (Maremonti et al., 2019). Nevertheless, the damage accumulated throughout whole developmental exposure seemed to be more critical towards reproductive outcomes (35% decreased broodsize associated with sperm count decrease), as also found by Maremonti et al. We hypothesized that exposure during the whole development induces an accumulation of damage, notably during gametogenesis, that are beyond possible repair and induce increased effects, i.e., sperm decrease and ovulation rate decrease, compared to early exposure. At this point however, the mechanisms underlying these effects were still unknown. These findings suggest that continued exposure induces additional damage, raising the importance of studies across several generations to increase knowledge on more ecologically relevant exposure situations for ERA.

5.1.2 Lipids as molecular markers of reproduction decrease?

Because lipids are target molecules of irradiation (e.g., lipid peroxidation via oxidative stress) and play an important role in reproduction processes as well (Hansen et al., 2013), we sought to characterize the response of lipids upon chronic exposure to IR in the nematode. We hypothesized that the quality rather than the quantity was more informative for understanding the precise mechanisms.

Cross analysis of lipid metabolism upon exposure to IR at different life stages revealed that the regulation of lipid metabolism was life-stage dependent just like reprotoxic effects. In particular, polar and neutral lipid contents were oppositely regulated upon embryogenesis exposure vs whole developmental exposure. This result indicated that opposite mechanisms

of regulation occurred at different life stages. We identified these mechanisms to be due to opposite signals from the soma and the germline because of (1) the differences in the development of the germline vs somatic tissues in embryos vs older larvae, and (2) the differences in lipid signaling between the soma and the germline. Indeed, for the first point (1), during embryogenesis the germline consists of only two primordial germ cells, which quantitatively represents a small portion of the whole organism compared to somatic tissues. Moreover, somatic cells have a better repair mechanism of DNA damage than germ cells and thus tolerate higher levels of IR than germ cells (Vermezovic et al., 2012, Clejan et al., 2006, Lemmens and Tijsterman, 2011). We thus suggested that germ cells of the irradiated embryos would accumulate more damage (including DNA damage) than somatic cells, and induce early germline deficiency signaling. Concordant with this hypothesis, previous studies have shown that germline defective mutants and sterile mutants exhibited an increase in neutral lipid content (Kuzmic et al., 2019, Hansen et al., 2013), which we found in irradiated embryos. On the contrary, worms irradiated throughout whole development showed a decrease in neutral lipid content. This was supported by other studies showing that oxidative stress induces lipid catabolism, in particular via autophagy (Maremonti et al., 2020c, Steinbaugh et al., 2015, Ezcurra et al., 2018). In line with our second point (2), this mechanism is mainly regulated via somatic signals (i.e., intestine). Thus, these findings suggest that while somatic cells do not seem to be affected during embryogenesis exposure, whole developmental exposure would induce sufficient damage to alter the signaling pathways in the soma. This early damage to the germline could be linked to the 11% broodsize decrease that we observe upon exposure throughout embryogenesis. Additionally, whole developmental exposure induced an increase in polar lipid content. This was associated with previous results showing an increase of vitellogenins expression (transporters of yolk granules from the intestine to the germline). This suggested a defective germline not able to uptake yolk granules leading to an accumulation of those in the soma.

In summary, cross analysis of lipid metabolism and life-stage sensitivity enabled us to understand the different signaling pathways induced by both the soma and the germline in response to chronic IR exposure. It also enabled us to understand that a misregulation of lipid metabolism is not necessarily a cause of reproduction decrease, but rather that their connection is intricate and complex, with many feedback loops, as shown in the proposed

conceptual model in Figure 17 (see Paper I p.86). Nonetheless, these results made it possible to increase the weight of evidence of previous findings (e.g., vitellogenin increase, damage in the germline), an essential criterion for AOP development. At this point, no explanation could yet be given for the mechanisms underlying the reduction of sperm count which appears to be the limiting factor regarding reproduction decrease.

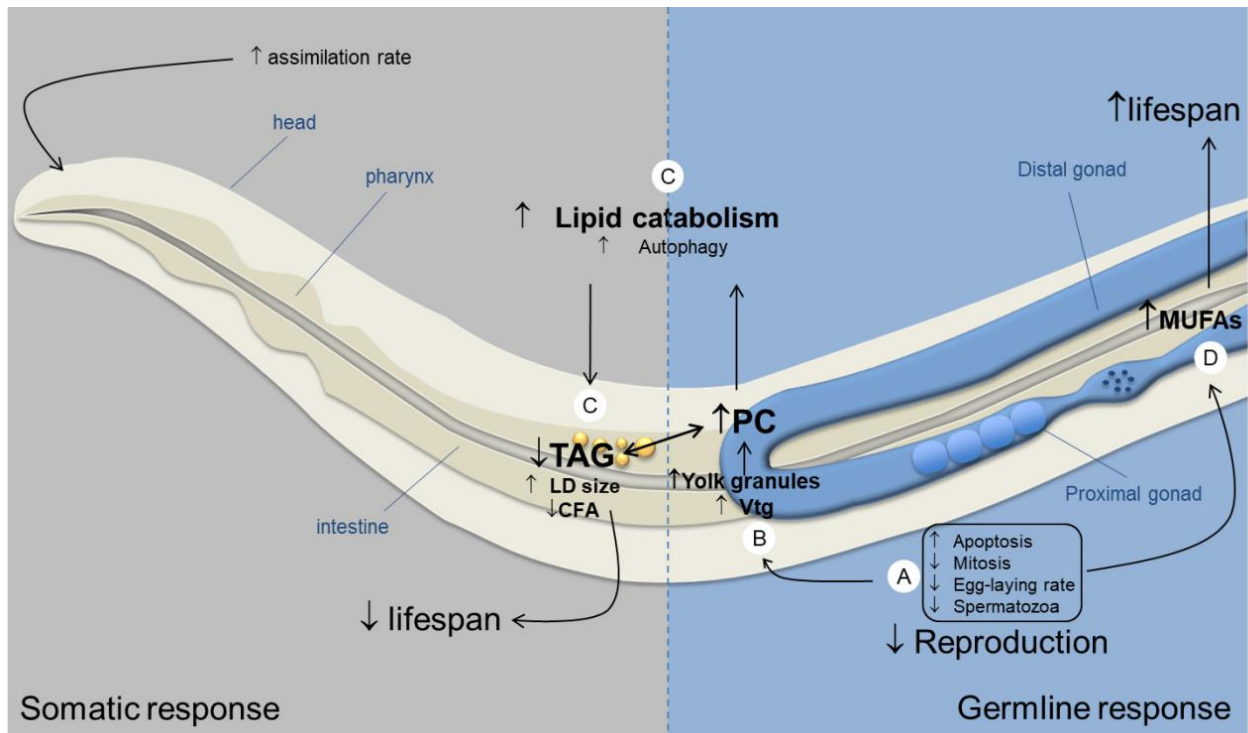


Figure 17 Proposed model for germline and soma regulation of lipid homeostasis after chronic exposure to IR during whole development in *C. elegans* N2 hermaphrodite (↑ corresponds to an increase, ↓ corresponds to a decrease). See Paper I p.86 for details.

5.1.3 Male and female gamete differences in radiosensitivity

In this project, the third objective was to assess the impact of gamete defects in the reproduction decrease, and in particular the relative contribution of sperm vs oocyte defects for the hermaphrodite. Several studies show the particular radiosensitivity of sperm compared to oocytes in *C. elegans* and other species such as earthworms (Hertel-Aas et al., 2007), fish (Kuwahara et al., 2002) and rodents (Liu et al., 2006). However, the relative impact of sperm decrease on reproduction decrease is highly dependent on the reproductive strategy. For example, in the earthworm, spermatogenesis is continuous and thus not a limiting factor (Hertel-Aas et al., 2007). In contrast, *C. elegans* spermatogenesis leads to a fixed number of spermatozoa, over a predefined period, then the germline switches to oogenesis

to produce oocytes during ~6 days. Self-fertile nematodes will then produce a number of offspring that is equal to the number of sperm available. Logically then, a reduction in sperm number will inevitably lead to an equivalent decrease of broodsize (Singson, 2001, Cutter, 2004, Murray and Cutter, 2011). However, we observed a decrease of broodsize upon chronic exposure to IR that was superior to the decrease of sperm number (35% vs 23%, respectively), and even noted a slight broodsize decrease in early stages exposed (11-12%) without a reduction in sperm number at all. This led us to believe that oocytes contributed, to a certain extent, to this broodsize decrease. Indeed, previous findings had already shown an increase of apoptosis upon chronic exposure to IR in the nematode (Maremonti et al., 2019, Buisset-Goussen, 2014), as well as an increase in the expression of vitellogenins, probably linked to defects in oocytes (Dubois et al., 2019). Moreover, since the apoptotic machinery is not recruited during spermatogenesis or in differentiated spermatozoa (Jaramillo-Lambert et al., 2010) but it is in oocytes, we assessed the effects of apoptosis on ovulation rate to determine if and how much it contributes to the ovulation rate decrease previously observed upon irradiation. Surprisingly, we found that the decrease in ovulation rate is attributed fully to increased apoptosis. This finding showed that ovulation rate decrease contributes to ~10% of total broodsize decrease vs 90% for spermatozoa.

A more direct approach to understand the relative contribution of sperm vs oocytes in this broodsize decrease would have been to cross males with female mutants (e.g., *fog-2* mutants). However, the reproductive strategy of males was difficult to compare to hermaphrodites. First, males produce an unlimited number of sperm, which, crossed with females producing oocytes continuously, would have made the count very fastidious and time consuming. Second, male sperm is morphologically different from hermaphrodite sperm (Singson, 2001), and the effects of IR on male sperm had never been assessed, so the extrapolation seemed risky. Besides, nematode populations in the environment only comprise ~0.1% of males, while the rest are hermaphrodites (Frézal and Félix, 2015), so it was more ecologically relevant to focus on hermaphrodites. In addition, the genes modified to generate female mutants are genes controlling the sex determination pathway (e.g., *fog-*, *tra-*, *her-* genes), which we assumed to be involved in the response to chronic exposure to IR. Therefore, the interpretation of results coming from these mutants might not have been accurate in regard to our hypothesis.

Regarding the causes of sperm decrease, we initially hypothesized that it could be linked to a premature developmental switch from spermatogenesis to oogenesis. We assessed several pathways that we identified to be involved in both this transition and the response to environmental stress, namely sex determination via TRA-1 and Ras/ERK (MPK-1). According to our results, none of these pathways seemed to be involved in the reduction of sperm count. However, MPK-1 from the Ras/ERK pathway was overactivated in the germline upon exposure to IR, which we suggested to be linked to increased apoptosis, consistent with previous functional tests using *ced-3* mutants. On the other hand, several previous results upon exposure in the same conditions observed in L4/YA showed a defect in germ cell proliferation in the mitotic zone of the germline. As previously described, since *C. elegans* sperm production is time limited, a decrease of future sperm cell proliferation could directly lead to a decrease in differentiated spermatocytes. On the contrary, it does not seem to have any effect on oocytes, for which the rate decrease was shown to be entirely attributed to apoptosis. We thus suggest the existence of two distinct mechanisms, as shown in Figure 18, inducing a decrease in ovulation rate and sperm count, both contributing to a decrease of total broodsize.

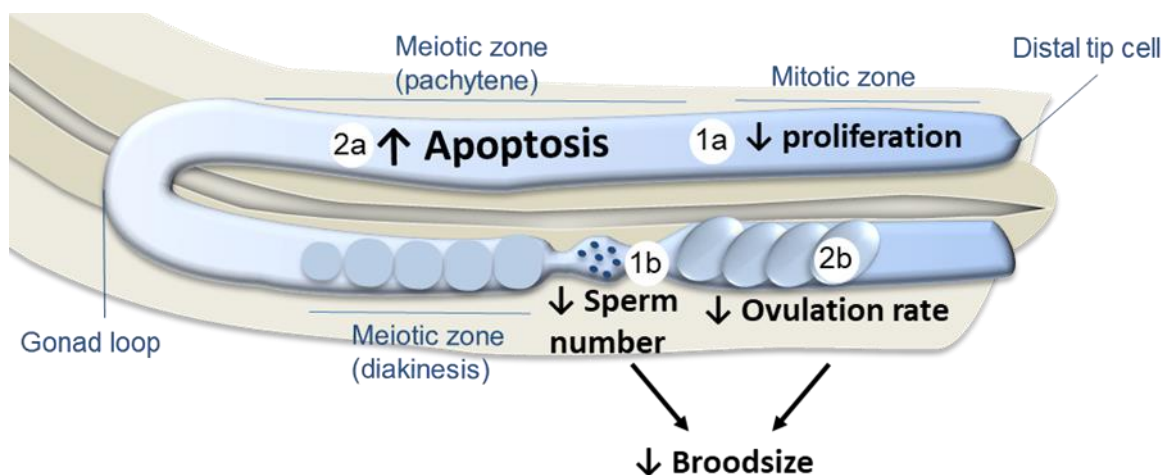


Figure 18 Proposed conceptual model for mechanisms causing broodsize decrease in hermaphrodite *C. elegans* exposed to chronic ionizing radiation. See Paper II p.106 for details.

Still, more direct evidence needs to be assessed to confirm this hypothesis of germline spermatocyte proliferation defect. For example, DNA imaging of proliferating cells (using for instance EdU staining in a pulse-chase analysis) could be interesting to assess the progression

into the cell cycle of germ stem cells upon exposure to IR, at a timing concordant with spermatogenesis. Other studies showed that sperm decrease could be linked to meiosis defects (Maremonti et al., 2019). Considering that a post-spermatogenesis irradiation on its own even at significantly higher doses (~ 15 Gy – < 100 mGy/h⁻¹ for 149h late L4 to end of reproduction) did not affect reproduction at all, the authors suggested that the process of spermatogenesis is more sensitive than the sperm maturing process. Consistent with this postulation, they found a dysregulation of genes involved in *C. elegans* spermatogenesis, in particular during chromosome segregation in meiosis (*smz-1* and *smz-2*) and chromatin condensation during sperm maturation (*htas-1*). The authors propose a model in which spermatogenesis is impaired via DNA damage causing defective chromosomal segregation and spindle formation during sperm meiosis (Maremonti et al., 2020b). However, a study from Guédon et al. (2021) showed a significant decrease in the activation of spermatids to spermatozoa in exposed nematodes, suggesting that both quantity and quality of sperm is affected by IR. Defect in sperm activation could either be the consequence of a yet unknown mechanism or only the consequence of MSP decrease, a sperm protein required for sperm activation. MSP was previously found to decrease upon chronic exposure to IR, most probably due to a reduction of sperm quantity (Guédon et al., 2021). However, since no effect on the viability of embryos (hatching success) was observed and spermatocytes do not experience apoptosis (similar reduction of sperm number with or without apoptotic pathway engaged), it seems that no unviable sperm have fertilized oocytes and that unviable sperm, if there are any, are somehow eliminated via another mechanism than apoptosis, respectively.

Despite all the knowledge gaps that were filled during this project, molecular mechanisms underlying reprotoxic effects could still be refined. In particular, the mechanism resulting in sperm decrease remains to be deciphered. To date, two non-mutually exclusive hypotheses need to be confirmed: quantitative assessment of germ cell proliferation decrease and understanding of meiosis impairment. In addition, considering that the quality of sperm also seemed to be affected by IR (Guédon et al., 2021), it would be interesting to understand if this contributes to the sperm number decrease, and if so, how they are eliminated. Analysis of spermatid morphology and viability could help to target the potential faulty mechanisms.

However, while our study focused on one generation irradiated, other projects in the lab have focused on assessing the effects of IR exposure directly at the population scale, i.e., on life-history traits of chronic exposure to IR over 25 generations of nematodes (Quevarec et al., submitted). The authors observed an increase of male frequency in the population of about 20%. Although outcrossing with *C. elegans* males induces an increase of total broodsize (up to 1200-1400 progenies per individual)(Anderson et al., 2010), the authors still observed a decrease in population size by 2%. This was due to the fact that hermaphrodites, not crossed with males, showed a decrease of broodsize of about 26%. It therefore appears that the reprotoxic effects in hermaphrodites that we observed on one generation are similar to those observed after 25 generations exposed, but the increase of male frequency over time rescues the population size. In *C. elegans*, the increase of male frequency under stressful conditions to enhance reproduction has been previously observed and is part of its reproductive strategy to favor outcrossing and resistance to stress (Cutter et al., 2003) (Ayyadevara et al., 2014, Kim and Rose, 1987). Even though the change in sex ratio seemed to rescue the population size, the cause of the slight decrease that is observed could be questioned. We can wonder if it might be due to the defects in oogenesis that we observed in this thesis, suspected to account for 10% of the broodsize decrease. This 10% reduction cannot be rescued by crossing with males and could thus be responsible for the 2% decrease observed after exposure upon 25 generations. Therefore, while basal mechanisms are easier to decipher on one life cycle, observations at one generation are not totally easy to extrapolate to population outcomes and a change of biological scale may imply new possible outcomes. This is one crucial point for environmental risk assessment and highlights the importance of multigenerational studies.

5.2 IMPLICATIONS FOR ENVIRONMENTAL RISK ASSESSMENT

Among the knowledge gaps identified for ERA and previously presented in the introduction, the data acquired in this project contributes to expanding the knowledge on sub individual effects that could help us to understand differences in species sensitivity to radiation. However, for the ERA framework, it is still necessary to link them to individual effects and beyond, if possible. We thus structured our data into an AOP scheme. In this part, we discuss the potential of our AOP scheme but also the limits of our data for application into the ERA framework.

5.2.1 Potential for ERA through the AOP framework

Within the AOP framework, we managed to characterize and causally link several key events that are essential for reproductive defects in the nematode *C. elegans*. The scheme is presented in detail in part 4.2. It enabled us to better assess the links between each event and identify the remaining knowledge gaps. To go further and build a better AOP scheme with stronger weights of evidence and better assess the essentiality of key events, we are currently crossing these data with other available data on *C. elegans* from the CERAD (Center for Environmental Radioactivity, NMBU Norway). This is done by developing the AOP#396, available on AOP wiki, jointly with some researchers from the CERAD.

This AOP could be extended to the nematode taxon, and probably to other species with similar reproductive biology (e.g., protandrous hermaphrodite, oviparous). We have identified common KEs across different taxa, such as an increase in vitellogenin synthesis in fishes upon exposure to certain chemicals (Doering et al., 2018, Villeneuve, 2018) and differential regulation of fatty acids in the crustacean *Daphnia magna* upon exposure to IR (Song et al., 2020). Still, the connection of these events with reproduction decrease in the nematode is unclear (see 4.2). However, the fact that these KE occur in other species with similar reproductive biology, i.e., oviparous, and are successfully linked with reproduction decrease in other AOPs can be clues for next steps to focus on and fill knowledge gaps in our own AOP.

At higher levels of organization, reproduction decrease usually leads to a decline in population. However, recent data on *C. elegans* suggested that the change in demographic structure (i.e., sex ratio) was more significant than global change in population size (Quevarec et al., submitted). It is not yet clear whether this change in sex ratio is beneficial or not for the population in terms of resistance to stress. Indeed, outcrossed offspring benefit from better fitness in changing environments (Prahlad, 2003). However, outcrossing with males appears to affect the survival and lifespan of hermaphrodites (Anderson et al., 2010; Maures et al., 2014; Cutter et al., 2019). Additional studies would be needed to clearly understand the consequences of this sex ratio change and link this effect with the reproduction decrease observed.

Since KEs are defined as relatively easy to measure, they could be used as *in situ* biomarkers for ERA, provided that they are linked with phenotypical endpoints (Lee et al.,

2015). In our AOP, an example of an easily measurable event successfully linked to reproduction decrease is 'increased apoptosis in oocytes', which could be measured via the level of expression of *egl-1* (via qRT-PCR for example). However, other effects contributing to reproduction decrease (i.e., sperm decrease) are not so easy to measure as they are quite fastidious (manually counting sperm via microscopy). Thus, for *in situ* purposes, better KEs must be identified to provide a more efficient way of measuring effects, or better ways of measuring KEs (such as automated methods). Interestingly, among the KEs included in our AOP, the vitellogenin is already used as a biomarker and endorsed by the OECD, notably for endocrine disruptors in fish species (Matozzo et al., 2008, OCDE, 2009). However, the link between vitellogenin expression and reproduction decrease in *C. elegans* is still unclear. This could be a next step to focus on. As a first step, some studies have already been initiated to understand the dose-response relationship of vitellogenin expression, and other proteins, using the DRomics tool (Larras et al., 2018). Determining dose and dose-rate threshold values would increase the utility of these data for ERA purposes. Therefore, a next step for our AOP could be to assess these links, considering the requirements for building a qAOP (Perkins et al., 2019).

Although the integration of this data into the ERA methodology is facilitated through the AOP construction, it is also important to highlight some limits of our approach.

5.2.2 Limits of the present data and domain of applicability

As previously stated, this AOP could be extended to species with a similar reproductive biology. This also represents a limit, as these results cannot be extrapolated to any species. For example, in nematodes, sperm is a limiting factor for reproduction, contrary to other taxonomic groups where oocytes are limiting (e.g., mammals). More knowledge is needed to understand the commonalities and discrepancies among species reproductive strategies, which is part of the objective of the current AOP Working group on radioinduced reproduction decline.

Moreover, the present data was collected in the conditions of chronic external gamma irradiation while, in the environment, species can be exposed to several radionuclides both internally (by ingestion, inhalation, etc.) and externally. To date, the estimation of internal dose in wildlife is a major uncertainty in environmental assessment (Beresford et al., 2008,

Beresford and Howard, 2014, Gómez-Ros et al., 2008). In this study, we used the γ -emitter Cs-137 which contributes mainly to external dose (Cunningham et al., 2021). Although it is relevant in terms of radionuclides found in the environment after accidental exposure (>30% of radionuclides in the Chernobyl Exclusion Zone)(Beresford and Copplestone, 2011), radionuclides other than γ -emitters may significantly contribute to the total absorbed dose, via internal dose, depending on the radiological context (mainly γ -emitters in Fukushima) (Beresford et al., 2020, Cunningham et al., 2021). In terms of effects, because γ rays have a high penetrating power, it might not be necessary to consider the internal contamination of γ -emitters. However, characterizing internal contamination from α and β emitters would be relevant as α and β rays do not penetrate deep in tissues, but they produce different types of effects locally (more direct damage than indirect damage, contrary to gamma rays).

In this study, we use the model species *C. elegans*, which is known to be quite radiotolerant (Hartman et al., 1988). Thus, the lowest dose rate needed to induce individual reprotoxic effects on the first generation was 50 mGy.h⁻¹ which is much higher than the dose rates currently found in contaminated areas. Indeed, it was found that *in situ*, terrestrial animals are unlikely to be detrimentally affected at dose rates <1 mGy.d⁻¹ (Beresford and Copplestone, 2011). However, in this project the use of *C. elegans* was essential to characterize molecular mechanisms of response to IR, considering the existing biological knowledge of this species. In addition, the study of molecular mechanisms in the nematode revealed a lower sensitivity threshold than for individual effects, as for example proteome modulation occurring as low as 0.5 Gy (~7mGy.h⁻¹) (Dubois et al., 2019) for some proteins possibly linked to reprotoxicity. It is crucial to collect more data on invertebrates, considering their importance in the functioning of ecosystems and the fact that they are estimated to represent 97% of total species on the planet (Eisenhauer et al., 2019). Currently, they represent less than 7% of species covered by radiation effects studies in the FREDERICA database (Copplestone et al., 2008).

Finally, by comparing our data with results obtained after 25 generations irradiated (Quevarec et al. 2022), we noticed that the final adverse outcome is not the same, i.e., no significant change in population size, but rather a change in sex ratio. This calls into question the changing scale issue.

5.2.3 Changing scale: an important challenge for ERA

Understanding sub individual mechanisms responsible for reproduction decrease is essential to characterize the determinants of species sensitivity and define more sensitive threshold values for protection of ecosystems to predict toxicity of pollutants. However, the integration of this type of data into the ERA framework is challenging because it does not necessarily reflect the effects on populations and higher biological systems (i.e., communities, ecosystems), which are concerned by protection efforts. When units of biological material are put together, the properties of the new material are not always additive, or equal to the sum of the properties of the components. Instead, at each level, new properties and rules emerge that cannot be predicted by observations and full knowledge of the lower levels. Such properties are called emergent properties (Novikoff, 1945, Morowitz, 2002). For example, at the community level, interactions between species can alter the adverse outcomes within populations (e.g., prey-predator relationships (CLEMENTS et al., 1989, McClellan et al., 2008)). Even at the sub individual level, emergent properties are necessary to understand the complexity of a system (e.g., from one cell to a multi-cellular system, organ or tissue). In order to better assess effects when changing scales, it is thus necessary to 1) causally link effects at different scales and 2) consider emergent properties within the new biological scale. The latter is a significant challenge that makes it difficult to integrate data at the community/ecosystem level into the AOP framework, and predictive models in general which are meant to be simplistic (Knapen, 2021, Forbes and Calow, 2012). As is, one of the biggest challenges that remains is to find the balance between simplistic and efficient tools for decision makers and more complex tools that accurately depict the reality of life.

Due to these differences, lab data are not easily extrapolated to field conditions. Field studies are thus still necessary to accurately assess effects on individuals and populations in a realistic ecological context (Burraco et al., 2021).

SCIENTIFIC PAPERS

PAPER I

Article

Deciphering differential life stage radioinduced reproductive decline in *Caenorhabditis elegans* through lipid analysis

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Abstract: Wildlife is chronically exposed to various sources of ionizing radiations, environmental or anthropic due to nuclear energy use, that can induce several defects in organisms. In invertebrates, reproduction, which directly impacts population dynamics, has been found to be the most radiosensitive endpoint. Understanding the underlying molecular pathways inducing this reproduction decrease can help in predicting the effects at larger scales (i.e., population). In this study, we used a life stage dependent approach in order to better understand the molecular determinants of reproduction decrease in the roundworm *C. elegans*. Worms were chronically exposed to 50 mGy.h⁻¹ external gamma ionizing radiations, throughout different developmental periods, namely embryogenesis, gametogenesis, full development. Then, in addition with reproduction parameters, we performed a wide analysis of lipids (different class and fatty acid via FAMES), which are both important signaling molecules for reproduction and molecular targets of oxidative stress. Our results showed that reproductive defects are life stage dependent, that lipids are differently misregulated according to the considered exposure (e.g., upon embryogenesis and full development) and do not fully explain radiation induced reproductive defect. Finally, it enables us to propose a conceptual model of lipid signaling after radiation stress in which both the soma and the germline participate.

Keywords: Ionizing radiation; oxidative stress; lipids; reproduction; *Caenorhabditis elegans*; chronic exposure; life stage

1. Introduction

Ionizing radiations (IR), originated from natural radiation and anthropic activities, induce a continuous exposure of wildlife species requiring the acquisition of specific data to understand the chronic effects and improve ecological risk assessment of IR. To date, knowledge still needs to be increased since radiosensitivity of environmental

species covers a wide range of dose rates without any explanations [1, 2] and risk assessment still uses data coming from acute exposure even though mode of actions and response mechanisms can be different than for chronic exposure [3-5]. Reproduction, which directly impacts population dynamics, is the most radiosensitive individual parameter in many invertebrates [6-10]. Especially, in the roundworm *Caenorhabditis elegans*, chronic exposure to IR during whole development causes a reproduction decrease (upon 50 mGy.h⁻¹) [4, 11] still unexplained fully. The understanding of mechanisms underlying the radioinduced reprotoxic response of *C. elegans* under chronic exposure scenarios is therefore of main concern.

Biological effects of IR are mainly due to oxidative stress which is caused by water radiolysis inducing the production of free radicals and reactive oxygen species (ROS) in cells [12, 13]. The accumulation of ROS in the organism after prolonged exposures to radiations has the potential to alter biological pathways [14-16], some of which are also involved in the regulation of lipid metabolism [17-21]. Specifically, IR mediated oxidative stress induces lipid peroxidation and neutral lipid storage in several species [22-24], as well as the misregulation of proteins linked to lipid transport (i.e., vitellogenins) in *C. elegans* [3].

In *C. elegans* as in many species, lipid metabolism and reproduction are interconnected [17, 25-32]. Thus, each lipid class might have some dedicated roles, signaling and regulations in reproduction processes. However, current studies on the effects of IR on lipids mainly focused on the regulation of total lipid content [22, 23, 33] although the understanding of compositional changes in lipid classes and fatty acids should give better information on the mechanisms occurring after a stress [34, 35].

Additionally, effects on organism reproductive capacity can have several origins including damages on stem cells, gametes and embryos, occurring at different life stages of development [36-40]. In *C. elegans*, life stage sensitivity partly depends on their constitutive cell types (e.g., somatic, germ cells, meiotic, post mitotic). Indeed, somatic and germ cells have different tolerance to radiation damages due to their differences in division rate and repair mechanisms [41-49].

To decipher the developmental stage that yields reproduction decline, we identified 3 critical developmental periods that could be particularly sensitive to radiations:

- Embryogenesis (*in utero* to L1 stage) during which gonad precursors cells are formed;
- Early development (until late L3 stage) until the beginning of meiosis during which the gonad cells (somatic and germ cells) divide rapidly;
- Gametogenesis (late L3 stage – Young adult (YA)) during which germ cells begin and achieve meiotic divisions.

In our study, *C. elegans* N2 strain, were chronically irradiated *in utero* until the end of aforementioned stages at the same dose rate of 50 mGy.h⁻¹, in order to respect the chronic exposure scenario and dose rate from previous studies [3, 11, 24]. All the worms were analyzed at the L4-YA stage. Reproduction parameters, class of lipids and fatty acids (FA) were assessed for each condition.

2. Results

2.1 Chronic radio-induced reprotoxic effects are life-stage dependent

In a first step, global reproduction endpoints such as brood size and hatching success were assessed at L4-YA stage. Figure 1A shows a brood size decrease for all scenarios compared to controls (-35% for SC3, $p = 2.65 \times 10^{-13}$, -11% for SC1, $p = 0.0195$, -12% for SC2, $p = 0.0130$), with an increased effect for SC3 compared to SC1 and SC2 ($p < 0.001$ – Tukey contrasts with Bonferroni correction). On the contrary, no significant differences between scenarios were found on the hatching success (Figure S1). To go further, egg laying rate (Figure 1B) and spermatid numbers (Figure 2) were assessed in

our conditions. Figure 1B shows a decrease in egg-laying rate for SC3 ($p = 2.45 \times 10^{-5}$) and SC2 ($p = 0.0104$) compared to controls, and between SC3 and SC1 ($p = 0.0105$ – Tukey contrasts with Bonferroni correction). Figure 2A shows a decrease in mean spermatid number compared to controls only for SC3 (-23%, $p < 1e-05$). These results altogether mean either an amplification in SC3 of an effect occurring during early development (SC1 and SC2), or two distinct mechanisms, early and late responses causing a different decrease in brood size.

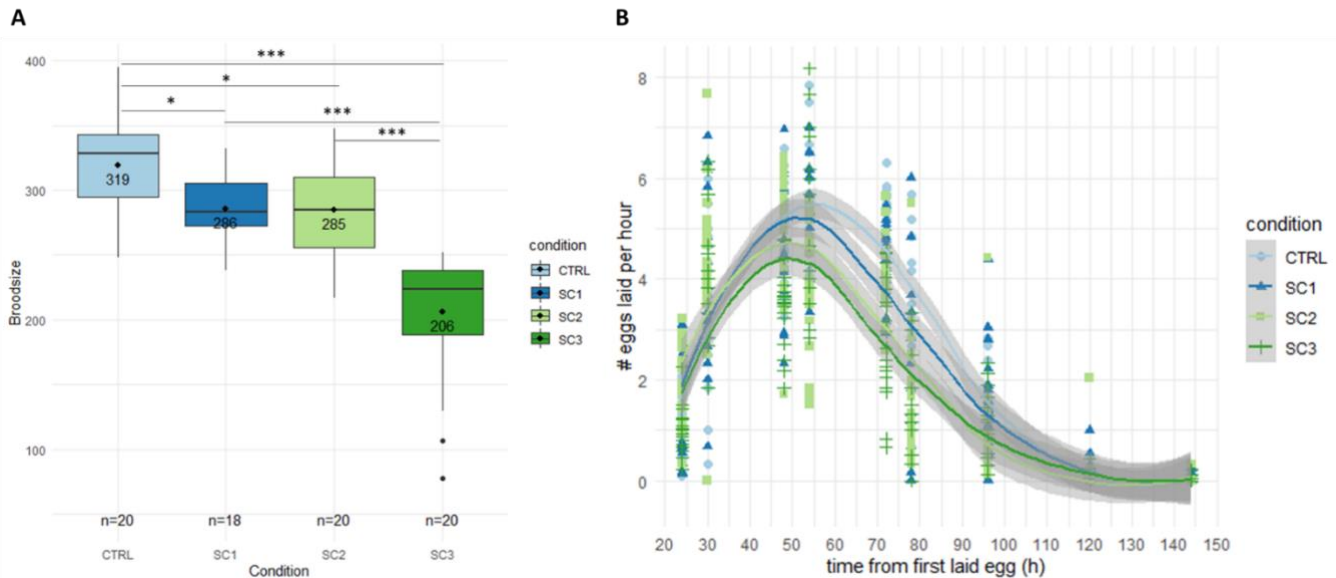


Figure 1. (A) Total broodsize during 6 days of egg-laying ($n=18-20$ per condition (SC1: embryogenesis, SC2: early development, SC3: full development), Generalized Linear Model (GLM) and Tukey *post-hoc* with bonferroni adjustment). (B) Egg laying rate (number of eggs laid per hour over 6 days) for each condition (smooth method is loess regression with 0.95 confidence interval) (significant differences were found between SC2-CTRL, SC3-CTRL, SC3-SC1, see text for p values)

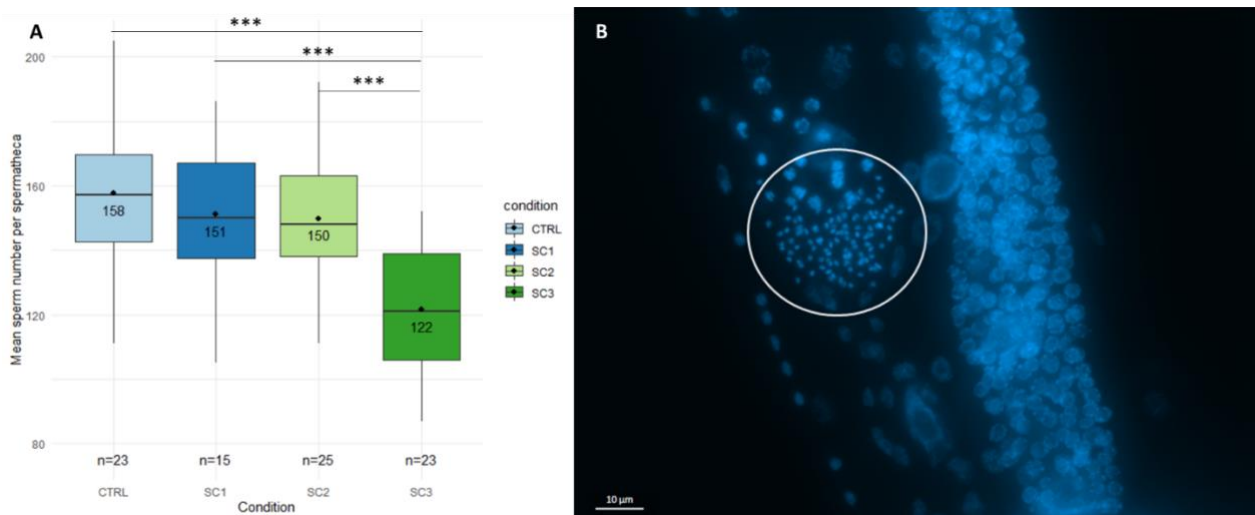


Figure 2. (A) Spermatid number per spermatheca ($n=15-25$ per condition (SC1: embryogenesis, SC2: early development, SC3: full development), GLM, Tukey *post-hoc* with bonferroni adjustment). (B) DAPI-stained *C. elegans* gonad (Maximum intensity projection of Z-stacks image. White circle shows the spermatheca containing spermatid-stained nuclei counted with FIJI, on the right of the spermatheca is shown the distal gonad with proliferating germ cells)

2.2 Lipid classes are differentially modulated according to the irradiated life stage

In *C. elegans*, only 7 to 20% of fatty acids (FA) are *de novo* synthesized, while the remaining are derived from bacterial food with a daily renewal of phospholipids [25].

In order to discriminate indirect effects coming from the irradiation of dead bacteria lipids' vs direct effects on nematodes, lipid content in nematodes fed with irradiated OP50 (OP50(i)) was also measured (see Figure A1).

Results of neutral lipids (triacylglycerols - TAG) and polar lipids (phosphatidylethanolamine - PE and phosphatidylcholine - PC) content in the worms are given in Figure 3.

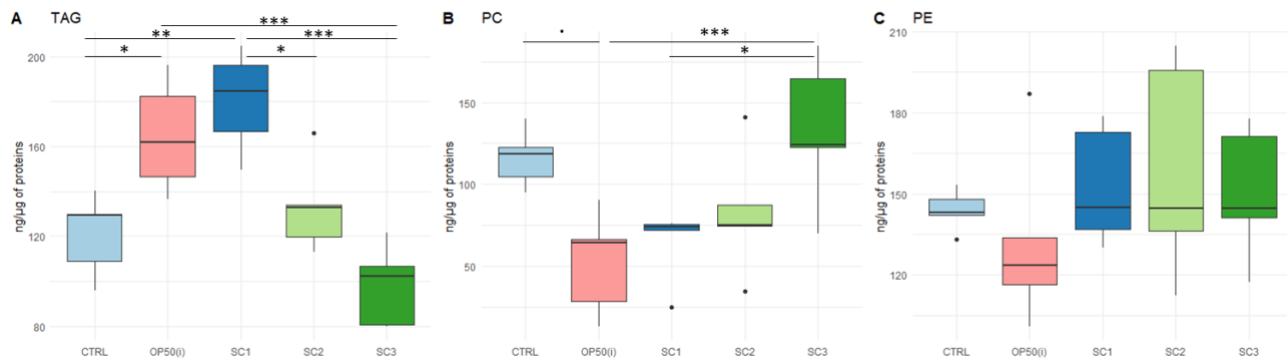


Figure 3. Lipid content per class (ng/μg of total protein) for each scenario (SC1: embryogenesis, SC2: early development, SC3: full development, OP50(i): control nematodes fed on irradiated OP50). (A) Triacylglycerols (TAG) (Anova; $R^2=0.66$, $F(4,20)=12.69$, $p=2.66 \times 10^{-5}$) (B) Phosphatidylcholine (PC) (Anova; $R^2=0.43$, $F(4,20)=5.61$, $p=0.0034$) (C) Phosphatidylethanolamine (PE) (Anova; $R^2=0.06$, $F(4,20)=0.67$, $p=0.62$). (All post-hoc tests are Tukey with bonferroni adjustment – p values from multiple comparisons are given in the text)

Neutral lipid analysis (Figure 3A) shows a significant increase in TAG content for worms fed on irradiated bacteria compared to controls ($p=0.033$). Thus, there is an effect of IR on bacteria inducing an increase of TAG content in nematodes. In contrast, there is a significant decrease in TAG content in SC3 compared to OP50(i) ($p=0.00062$), for which bacteria were irradiated in the same conditions as SC3. However, there is a significant increase in TAGs for SC1 compared to controls ($p=0.0021$), SC2 ($p=0.018$) and SC3 ($p=4.28 \times 10^{-5}$), SC1 being more comparable to controls regarding the duration of exposure of bacteria (Figure S1). Therefore, there is a significant increase in TAG content for SC1 but a decrease for SC3.

Polar lipids analysis (Figure 3 B-C) showed a decrease in PC content for OP50(i) compared to controls (significant with an α risk of 10%, $p=0.053$), suggesting an effect of irradiated bacteria inducing a decrease in PC content in nematodes. In contrast, there is an increase in PC content for SC3 compared to OP50(i) ($p=0.0077$) and SC1 ($p=0.031$). Therefore, there is an increase in PC content for SC3, and there seems to be a decrease in SC1 (compared to controls), however not significant. No significant difference was found for PE content.

Overall, spearman correlation analysis showed a negative correlation between TAG and PC ($\rho = -0.47$, $p < 0.05$).

2.3 FAMES analysis shows the radiation induced modulation of specific fatty acids

Figure 4 describes the fatty acids methyl esters (FAMES) content from C12 to C22, weighted by the amount of proteins, for all our conditions and shows a modulation of some specific fatty acids (FA) mostly for SC3 (PCA Figure S2).

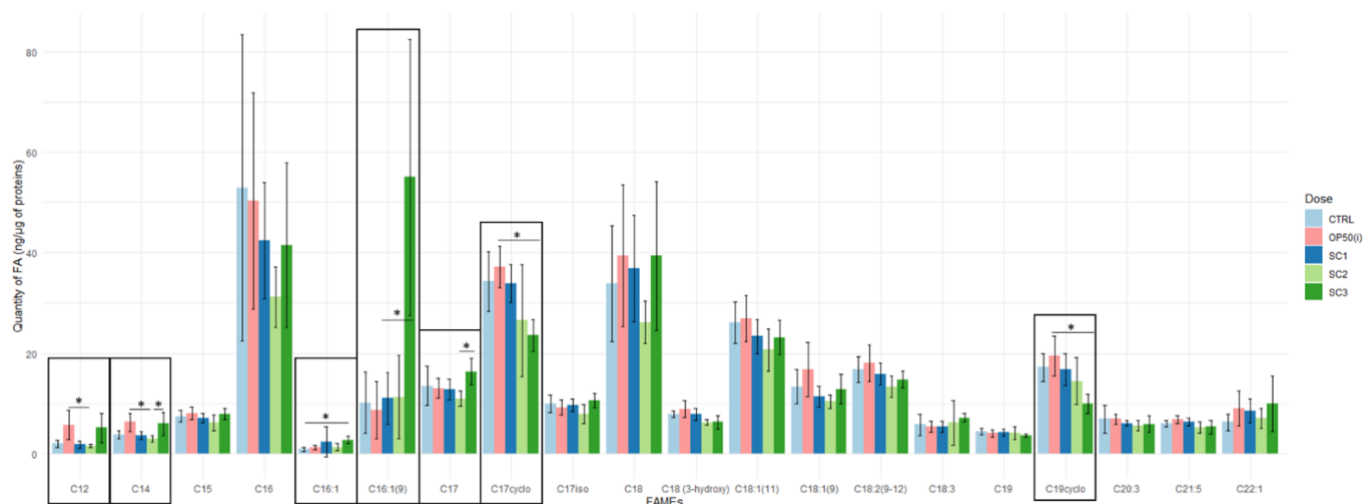


Figure 4. FAMES content ($\text{ng} \cdot \mu\text{g}^{-1}$ of total protein content) for each scenario ((SC1: embryogenesis, SC2: early development, SC3: full development), Kruskal Wallis, Dunn post-hoc with bonferroni adjustment)

In SC3, C16:1 compared to controls ($p = 0.0399$), C16:1 (9) compared to OP50(i) ($p = 0.0303$) and C17 compared to SC2 ($p = 0.0114$) were increased. While C17 Δ ($p=0.0034$) and C19 Δ ($p=0.0047$) seem to be decreased compared to OP50(i). In SC2, C12 ($p = 0.0263$) and C14 ($p = 0.0147$) decreased significantly compared to OP50(i). Spearman correlation analysis between total dose for each scenario and FAMES content (Table S2) showed a significant negative correlation for C17 Δ , C19 Δ , C19, 3-hydroxy C18 with the total irradiation dose (see Table S1) and significant positive correlation for C16:1, C16:1(9) and C12.

Among those, we focused on the decrease of cyclopropane fatty acids (CFA) C17 Δ and C19 Δ in SC3. Since these FA come from bacterial food and are considered as non-essential to *C. elegans* metabolism [25, 50, 51], their decrease could be due to a defect in food uptake. We tested this hypothesis through (i) the attraction behavior of irradiated worms towards the bacterial lawn (ii) their locomotion (thrashing assay) and (iii) the content of ingested and assimilated bacteria in the intestine (see Supplementary Material and Methods). No effect of irradiation was found on locomotion nor attraction towards bacteria (Figure S3 & S4). However, the measurement of bacteria content in the intestine showed that irradiated worms tend to have a faster assimilation rate, however not significant (Figure S5; $p=0.07$). Then the decreasing rate of these two CFA in SC3 seems to be linked to their use.

2.4 *In situ* changes of lipid morphology

To confirm the results obtained after lipid analysis, we investigated the relative area of lipid droplets (LD) in irradiated worms (SC3) compared to controls, through TEM images. We observed that irradiated worm tissues tend to be less resistant to the slicing procedure (red arrows in Figure 5A) which is usually a result of an increased fat content. The measure of LD surface (Figure 5B) showed a significant higher lipid droplet size in irradiated nematodes ($p < 2 \times 10^{-16}$).

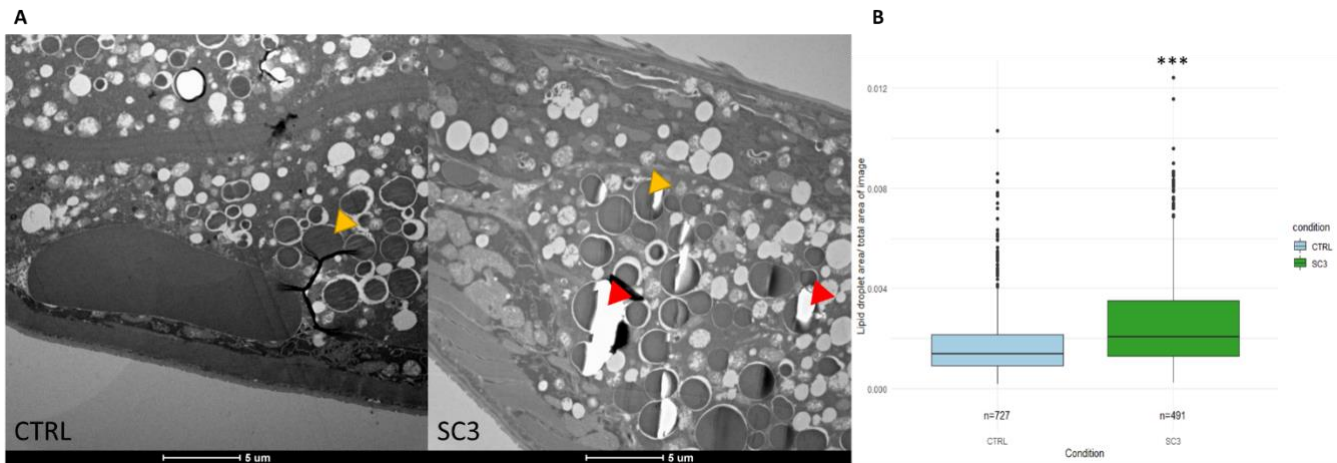


Figure 5. (A) Representative TEM images of *C. elegans* intestine (longitudinal) in each condition (Yellow arrows show the type of lipid droplets that were measured. Red arrows show tears in the slice. Images at similar zones, i.e. in the intestine and same scale were selected. See Figure S6 for example of acquisition method). (B) Lipid droplet surface measured in TEM images (7 individuals, i.e. 7 slices, were analyzed for each condition)

3. Discussion

Understanding the molecular determinants of chronic radiation response is of fundamental interest to predict effects at individual level. By studying the effects at different life stages and the changes in lipid composition we were able to better decipher the timing of occurrence and nature of these mechanisms.

3.1 Modulation of lipid content under stress: two opposite mechanisms controlled by the germline and/or the soma?

Analysis of lipid class revealed a negative correlation between TAG and PC levels and at different life stages (Figure 6). After exposure throughout embryogenesis (SC1), nematodes showed an increase in TAG content, the major form of long term energy storage, as is also found in germline-less or germline-deficient mutants [17]. During embryogenesis, only germ cell precursors are present and in a lesser amount than somatic cells. Somatic cells have been shown to have a better repair mechanism than germ cells and thus tolerate higher levels of IR than germ cells [42-44]. We suggest that under the SC1 scenario, somatic cells are not affected by IR because of the relatively low total dose or time of exposure (see Table S1), but germ cells precursors are, concordant with the TAG increase also observed in germline-less *glp-1* mutants. This could partly explain the 11% brood size decrease that we observe in SC1.

On the contrary, nematodes exposed throughout whole development (SC3) showed a decrease in TAG content, consistent with other studies showing an increase of lipid catabolism and autophagy [16, 19, 45] in *C. elegans* exposed to oxidative stress [17, 52], and with previous results in the same condition of exposure for germline-less *glp-1* mutants [24]. Lipid catabolism is controlled mainly by the soma [16] which suggests that somatic cells are affected in the SC3 scenario. In addition to this TAG decrease, we observed a trend of a faster food assimilation rate in the intestine which is concordant with previous findings through an energy-based modeling approach (DEBtox), suggesting that gamma radiation induces an increase in costs for somatic growth and maintenance in *C. elegans* [8]. This could translate to a faster assimilation of nutrients, resulting in the reduced TAG content and specific FA (CFA).

While TAGs decreased in SC3, nematodes showed an increase in PC content. Phospholipids are the main components of cellular membranes and yolk granules [25] composed at 85% of proteins and 15% of lipids. Yolk granules are synthesized in the intestine and transported in the germline through vitellogenins, for which we previously found an overexpression under the same conditions of exposure [3].

According to the literature, a deficiency in germline capacity to uptake yolk granules leads to an accumulation of those in the soma [26, 53] and, stressed or aging nematodes tend to consume their own intestine to enhance capacity for yolk production [19], which concurs with our present findings.

With these results, we suggest that IR induce an increase in lipid levels (PC and TAG) through germline signals, and a lipid catabolism (TAG) through somatic signals, leading to an unchanged global lipid level. Therefore, at least two opposite mechanisms exist in our conditions and contribute to balance total lipid content. Many studies have been addressing the issue of this double response to oxidative stress [17, 24, 26, 44], and its regulation by the germline [30, 52, 54], especially regarding the longevity endpoint [30]. However, rather than lipid level, it is the structural properties of FA such as chain length and saturation degree that may contribute to modulate processes such as longevity and reproduction [34, 35].

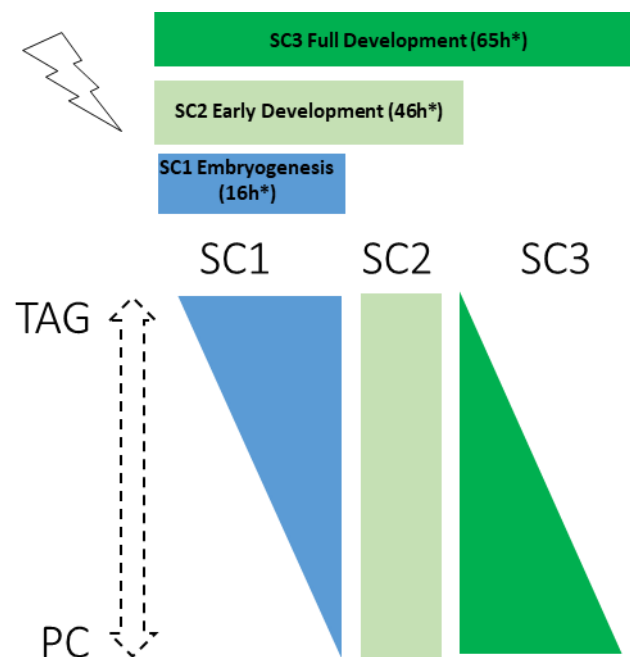


Figure 6. Continuous exposure to ionizing radiations induces life-stage dependent opposite regulation of PC and TAG contents

3.2 Facing radiation « all fat are not equal »: quality over quantity

Our results showed significant differences in FAMES mainly for nematodes exposed throughout their whole development (SC3). In particular, two C16 MUFA were increased, especially the palmitoleic acid (C16:1 (9)). Consistent with this finding, we also observed a decrease of CFA (C17 Δ and C19 Δ) which was previously reported to be linked to an increase in MUFAs [55]. MUFAs enhance membrane fluidity, reduce oxidative stress and contribute to energy storage and the activation of specific pathways [52] and is often associated to a longer lifespan [34, 56], especially in germline-less mutants [57]. However, no difference in lifespan was shown for the N2 strain in our conditions [8, 24] and, in the same conditions, germline-less *glp-1* mutants have even shown a decrease in longevity [24], suggesting once again two opposite balanced mechanisms responding to IR.

3.3 Reproduction decrease: deciphering mechanisms through lipid analysis

From lipid analysis, we suggest that the reduced brood size observed for nematodes exposed during embryogenesis (SC1) could be due to early unrepaired or misrepaired damages on germ cell precursors. Meanwhile, effects occurring in SC3 (i.e., more decreased brood size, egg-laying rate and sperm number decrease) occur concomitantly with other observed phenomena i.e., increased PC content and overexpression of vitellogenins [22], TAG catabolism, MUFA increase, CFA decrease and faster assimilation rate. In *C. elegans*, as in many species, egg quality and embryogenesis are conditioned by the activity of vitellogenins [53, 58-60] and were previously shown to be linked with LD size and their coalescence in the rainbow trout after IR [61]. Moreover, C17 Δ content, LD size and upregulation of vitellogenins seem to be linked to *daf-2* [62], involved in the Insulin-like signaling (IIS) pathway which, when negatively regulated, is associated with an increase in longevity and resistance to oxidative stress [18]. We found an increase in LD size and C17 Δ decrease in irradiated worms (SC3), which suggests that this pathway could be involved in the response to IR in our conditions [19, 51]. In addition, the upregulation of vitellogenins can also result from a DAF-2/IIS pathway regulation, notably through a sperm dependent signal [20], which is concordant with the observed sperm decrease in our conditions. In *C. elegans* hermaphrodites, many processes linked to oocytes such as meiotic maturation and ovulation are controlled by the MSP (Major Sperm Protein) found in sperm [63-65]. Therefore, a decrease in sperm number can directly affect egg-laying rate as observed in our study. Another cause of egg-laying rate decrease could be an increase of apoptosis [66] since an overexpression of *egl-1*, was previously observed in our condition of irradiation [11]. While oogenesis begins at stage L4 (included in SC3) and is constitutive during adulthood, sperm production occurs only during the late L3 stage (included only in SC3 here) and is limited to ~150 spermatids per spermatheca, meaning that nematodes will produce as many eggs as their sperm number unless mated with males [67-69]. According to the literature, sperm number could be controlled by protein myristoylation, in which the myristic acid (C14) plays a central role to regulate the spermatogenesis to oogenesis switch [70]. Indeed, an early switch could lead to a decrease in sperm number and premature oogenesis. In our study, no significant effect was found on C14, therefore it does not seem to be responsible for sperm number decline under IR exposure. Another hypothesis could be linked with cell-cycle arrest which was previously observed [8] and reduced mitotic cell number (data not shown), therefore limiting the pool of available germ stem cells to differentiate into gametes but this hypothesis was not investigated in this work.

Our results showed how both the germline and the soma could be involved in the regulation of lipid homeostasis. Thus we propose a conceptual model (Figure 7) for IR response in which the germline and the soma both act to regulate lipid content by signaling opposite mechanisms. Understanding the interplay between both cell lines enabled to shed light on the different observed effects on reproduction throughout life stages. Still, the question of sperm decline remains unsolved through the study of lipids.

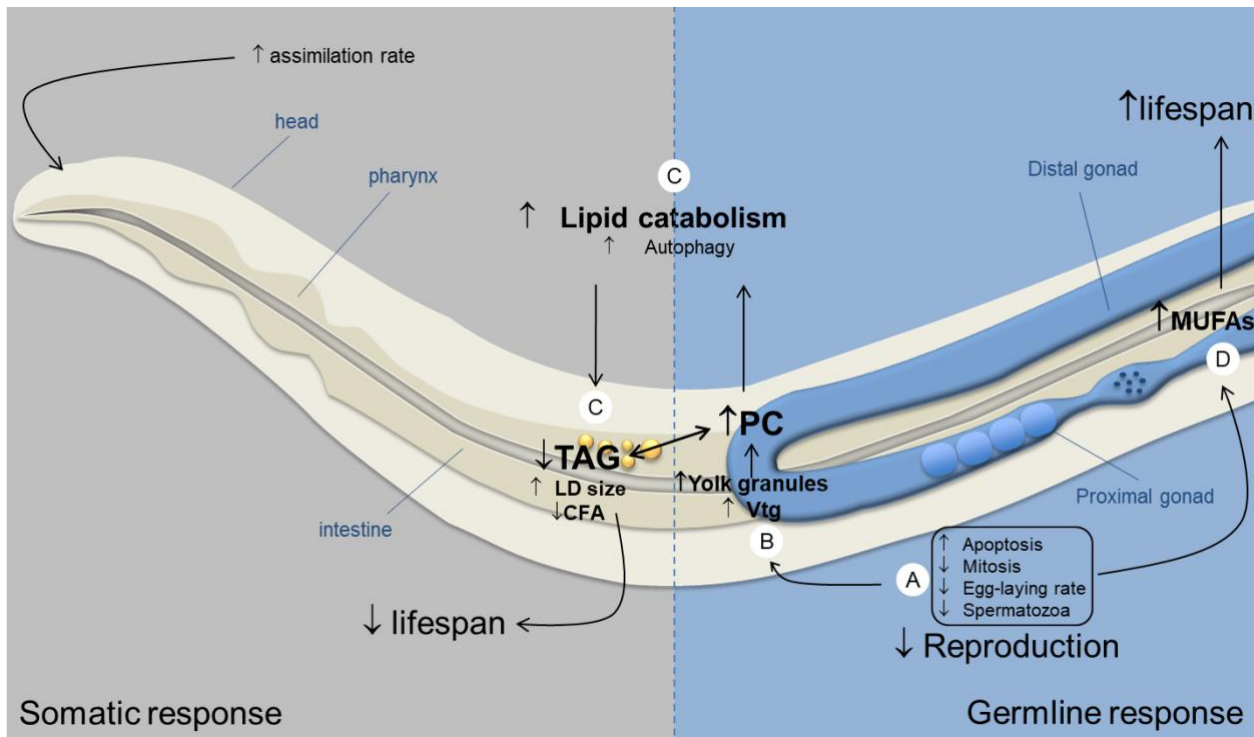


Figure 7. Proposed model for germline and soma regulation of lipid homeostasis after chronic exposure to IR during whole development in *C. elegans* N2 hermaphrodite (\uparrow corresponds to an increase, \downarrow corresponds to a decrease). IR induce germline defects (A) with the reduced egg-laying rate, brood size and sperm number (present study), the increase of radio-induced apoptosis and cell-cycle arrest [11] and reduced mitotic cell number (results not shown). This germline deficiency and/or the stress-induced intestine autophagy [19] could lead to (B) an accumulation of yolk granules as shown by the increased PC content (present study) and overexpression of vitellogenins (vtg) [3]. In turn, this accumulation of fat content could lead to lipid intoxication, known to reduce lifespan [71]. In response to this accumulation, the soma acts to regulate lipid levels by (C) increasing autophagy and lipid catabolism (probably through the IIS pathway) as shown by the decreased TAG and CFA content (present study), and the upregulation of genes involved in autophagy after IR exposure [16]. Autophagy is known to reduce lipid intoxication, thus enhancing lifespan [72]. However, under stress conditions, previous studies have shown that lipid catabolism was associated with decreased lifespan in germline less mutants [24, 32]. However, in N2 strains, longevity is not affected by IR under our conditions [8, 24]. Therefore, either lipid catabolism is also detrimental to lifespan, or the germline provides an opposite signal that induces an increase of longevity, compensating the positive response of the soma, as observed through the increase of MUFA content (D) (present study), known to be regulated by the germline [52, 54] and to enhance longevity [34, 35].

4. Materials and Methods

Strain and maintenance

The Bristol N2 *C. elegans* strain and *Escherichia coli* OP50 were provided by the Caenorhabditis Genetics Center (MN, USA). Nematodes were cultured at 19°C on Nematode growth medium (NGM) and M9 medium prepared by standard protocols [73]. Seeded plates with OP50 were prepared as previously described [11, 24].

Exposure to gamma-radiation and dosimetry

External gamma radiation exposure was conducted at the MIRE ^{137}Cs irradiation facility at the National Institute for Radioprotection and Nuclear Safety (IRSN) as previously described [11, 24].

Life-stage gamma irradiation experimental design

Nematodes were first exposed *in utero* through a population of gravid worms previously synchronized by bleaching [74]. After 8 h of exposure of the gravid worms, eggs already laid were separated from gravid worms by a sucrose gradient (3–7%), and gravid worms were bleached to collect the eggs *in utero* (synchronized over 3 h). Eggs

exposed *in utero* (~2000/replicate) were then replaced in the irradiation facility under the following 3 different scenarios of exposure (Figure A1):

- From *in utero* eggs to 16h post-ovulation, covering embryogenesis (until mid-L1 stage), *i.e.* SC1;
- From *in utero* eggs to 45h post-ovulation, covering early development until the beginning of meiosis (late L3 stage), *i.e.* SC2;
- From *in utero* eggs to 65h post-ovulation, covering full development (until L4-YA stage), *i.e.* SC3.

At the end of exposure scenario SC1 and SC2, nematodes were placed in recovery until they reached the L4/YA stage. All parameters were measured when nematodes reached the L4/YA stage. Total doses are given in Table S1.

Analysis of indirect effects of gamma radiation on bacteria

To assess the effects of irradiated bacterial food on lipid metabolism, another scenario (OP50(i)) was added to the experimental design. Non-irradiated nematodes were treated as controls but grown on previously irradiated bacterial lawn. The bacteria were irradiated, in triplicates, under the same exposure condition than SC3 (*i.e.* 65 h at 50 mGy.h⁻¹, Figure A1).

Assessment of reprotoxic effects

Reproduction assay

Cumulated larvae number and hatching success were quantified for each individual (20 per scenario) for 8 days. Nematodes were transferred to fresh NGM petri dish every 24 h. In addition, newly laid eggs were quantified every 6 h after the transfer every day. Hatching success was analyzed by counting the newly hatched larvae 24 h after the transfer.

Spermatids quantification

Samples were washed 3 times with M9 and 15 to 25 worms per replicate were mounted on slides pre-coated with Poly-Lysine (10 µL at 0.1%). Worms were immobilized with 3 µL of 0.1 mM levamisole and dissected using a 0.45 µm gouge needle, then fixed with 2% paraformaldehyde. The freeze-cracking method was used to remove the cuticle [75]. Slides were then fixed in methanol/acetone (1:1) for 20 min at -20°C, then rinsed 3 times in 1x-PBST. Slides were stained with DAPI mounting medium for 2h at +4°C in a dark chamber. Images were obtained with Axioobserver ZEISS Z1 microscope equipped with a DAPI filter system, at 40x and 12-bit resolution and Z-stacked for each spermathecal. Spermatids were quantified using the FIJI software [76].

Assessment of effects on lipid metabolism

At the end of exposure and recovery, samples were washed 3 times with M9 and redistributed as 5 replicates per condition (~1000 nematodes/replicate) for lipid and protein extraction. Samples were flash frozen in liquid nitrogen in a grinder tube with the minimal volume and stored at -80 °C until use.

Total lipids extraction

Lipids were extracted with a lysis buffer (acetic acid/EDTA 1mM) and zirconium grinding beads in a homogenizer (3x6500 rpm). Lipids in the aqueous phase were extracted by modified Bligh and Dyer [77]. Lipids in the organic phases were collected by evaporation under a nitrogen flow and adding of chloroform/methanol (2:1, v/v). One third of the volume was used for the analysis of neutral and polar lipids, while the remaining two thirds were used for fatty acid methyl ester (FAME) analysis.

Analysis of neutral and polar lipids

Polar and neutral lipid quantification was made by high performance – thin layer chromatograph (HPTLC) on a silica gel 60 (Merck). Acetone/toluene/water (91/30/8, v/v/v) was used as eluent for polar lipids and hexane/diethyl ether/acetic acid (17/3/0.2, v/v/v) for neutral lipids. Triheptadecanoin and heptadecanoic acid were used as neutral lipids standards. Polar lipids standards were phosphatidylcholine and phosphatidylethanolamine. The plates were revealed with orthophosphoric acid/copper sulfate (170°C for 20 min) then scanned at 500 nm using a TLC Scanner 3 with WinCATs software (Camag) [77]. Neutral and polar lipids content was weighted with total protein content extracted with the method described below.

Fatty acid methyl esters (FAMES) analysis

Fatty acids were transmethylated (sulfuric acid 5% in methanol, 1h30, at 85°C) into FAMES and extracted with hexane as described in Siaux et al (2011) [77]. Behenic acid was used as internal standard. Samples were injected in GC-MS-FID (Agilent) with Optima-Wax (0.25x30m) column and helium as vector gaz. The samples were split for detection: MS for identification and FID for quantification. FAME content was weighted with total protein content extracted with the method described below.

Protein extraction and quantification

After the separation of phases for lipid extraction, the aqueous phase was placed under nitrogen flow to evaporate residual solvents. 1% SDS was added, pH adjusted to 9 and placed under agitation for 1 h. Proteins were quantified using classical Bicinchoninic acid assay (BCA).

TEM analysis

After washing, ~500 worms per replicates (3 replicates per condition) were collected and fixed with 2.5% glutaraldehyde in 0.1 M, pH 7.4 sodium cacodylate buffer for two days at 4 °C. The samples were washed for 5 min three times with the same buffer. Samples were postosmicated with 1% osmium tetroxyde in cacodylate buffer for 1 h, dehydrated through a graded ethanol series, and finally embedded in monomeric resin Epon 812. All chemicals used for histological preparation were purchased from Electron Microscopy Sciences (Hatfield, USA). Ultrathin sections (70 nm; Leica-Reichert Ultracut E) were collected from different levels of each block, counterstained with 1.5% uranyl acetate in 70% ethanol and lead citrate and observed using a Tecnai F20 transmission electron microscope at 200 kV at the CoMET MRI facilities (INM, Montpellier, France). For lipid droplet analysis, 7 individuals (i.e. image) were analyzed for each condition using FIJI software [76].

Statistical analysis

All data were obtained with a simple random sampling. Count data (spermatids count, total broodsize, hatching success, egg laying rate and lipid droplet size) were analyzed using a GLM (General Linear Model). For lipid contents (FAMES, neutral lipids and polar lipids), when the data was normally distributed, means were compared using an Anova, otherwise, Kruskal Wallis test was performed. Specifically, a principal component analysis (PCA) and Spearman correlation analysis were conducted on FAMES. For each test, post-hoc analysis was conducted (Tukey and Dunnett for parametric tests, Dunn's for non-parametric tests) and p-values were adjusted using the bonferroni correction. An alpha risk of 5% was taken as significant. Statistical analysis was conducted on R Studio software, Version 1.1.423 (© 2009-2018 RStudio, Inc.), using the following packages for statistical tests: 'car', 'dunn.test', 'multcomp', 'FactoMineR', 'factoextra', 'psych', 'PerformanceAnalytics'.

5. Conclusions

Our study aimed to understand the link between reproduction decline and lipid metabolism after exposure to IR in *C. elegans*. In order to understand precisely when these mechanisms occur, we studied chronic exposure to IR at different life stages. Our results showed that different mechanisms occur at different developmental periods (embryogenesis, early development, full development), occasioning more or less severe response in reproduction, and opposite responses in lipid metabolism. We showed that these different responses could be due to opposite signaling from the germline and the soma, contributing to maintain lipid homeostasis. Numerous studies have focused on deciphering the mechanisms behind this double response, however it was unclear whether the lipogenic and lipolytic pathways act on the same or distinct types of lipids. In the present study, we showed that neutral and polar lipids, especially TAG and PC, are oppositely regulated after exposure to oxidative stress. In order to determine the links between these key events, we suggest that IR induce a defective germline which in turn leads to an accumulation of lipids (TAG) and favors the lipogenesis of specific fatty acids (MUFAs). In response to this misregulation, the soma acts as a negative regulator by inducing lipid catabolism (PC) to compensate this accumulation. Next studies will focus on deciphering the mechanisms behind this double response and the causes of reproduction decline.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Hatching success, Figure S2: Graph of individuals of the Principal Component Analysis on FAMES, Figure S3: Time course of attraction of nematodes to *E. coli* OP50 lawn for each condition, Figure S4: Number of thrashes for 30s for each condition, Figure S5: (A) Method for measurement of assimilation rate (Cy5-stained OP50 in the intestine). (B) Assimilation ratio for each condition, Figure S6: Example of analyzed image (CTRL) for lipid droplet area measurement using FIJI, Table S1: Cumulated doses for each condition, Table S2: Spearman correlation between total dose and FAMES content, and 'FAMES' variable contribution to PCA axis.

Author Contributions: Conceptualization & methodology, E.D.S. and S.F.; writing—original draft preparation, E.D.S.; writing—review and editing, S.F., Y.L. and S.G.; supervision, S.F. and S.G.; investigation, E.D.S., S.F., M.R., S.C. and L.Q.; formal analysis, E.D.S., S.F.; data curation, E.D.S., S.F.; visualization, E.D.S. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

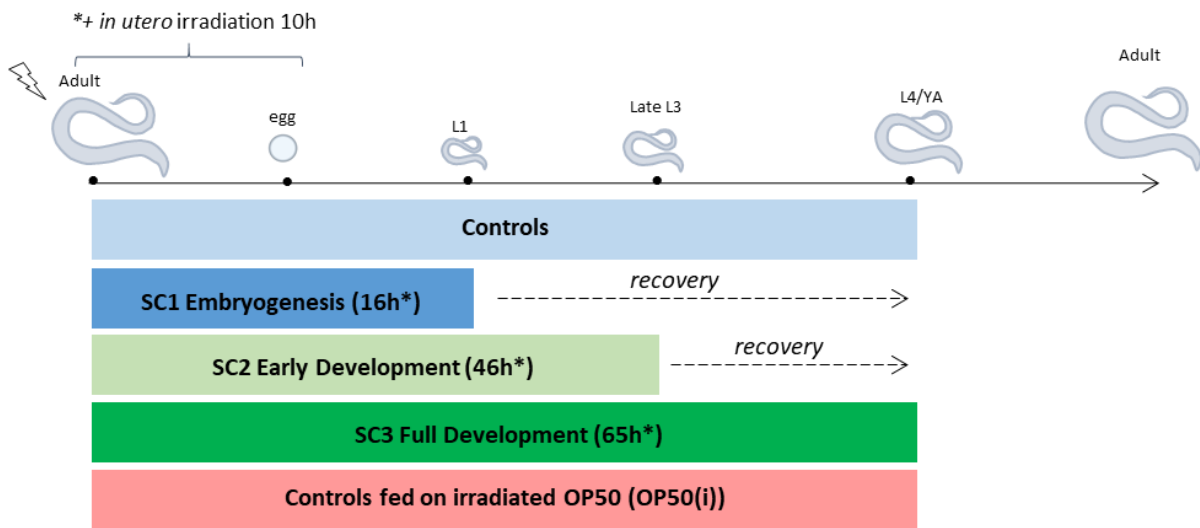


Figure A1. Experimental design for analysis of life stage dependent reprotoxic effects (SC: scenario, OP50: *E. Coli* strain, L1-L4: *C. elegans* larval stages, YA: Young Adult).

Supplementary Material

| Exposure Scenario | Cumulated Dose (Gy) (mean ± SD) |
|-------------------|---------------------------------|
| SC1 | 1,10 ± 0,2 |
| SC2 | 2,41 ± 0,2 |
| SC3 | 3,23 ± 0,22 |
| OP50 | 2,92 ± 0,21 |

Table S1. Cumulated doses for each condition

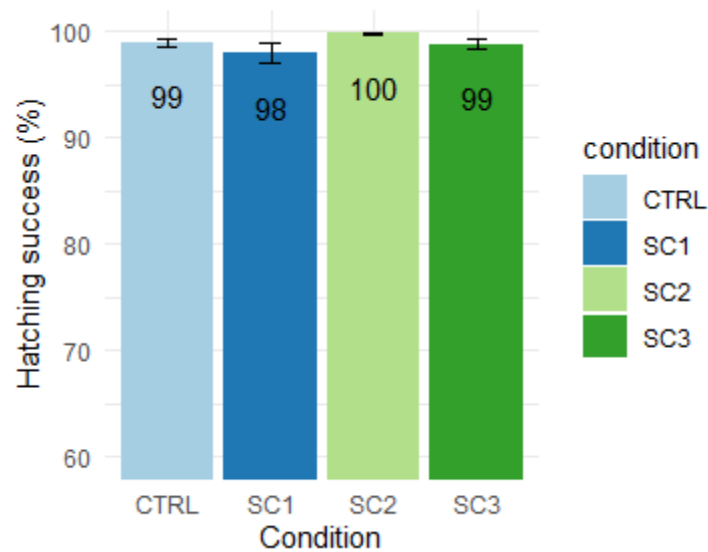


Figure S1. Hatching success (means ± se, n=18-20 for each condition - GLM on total unhatched eggs)

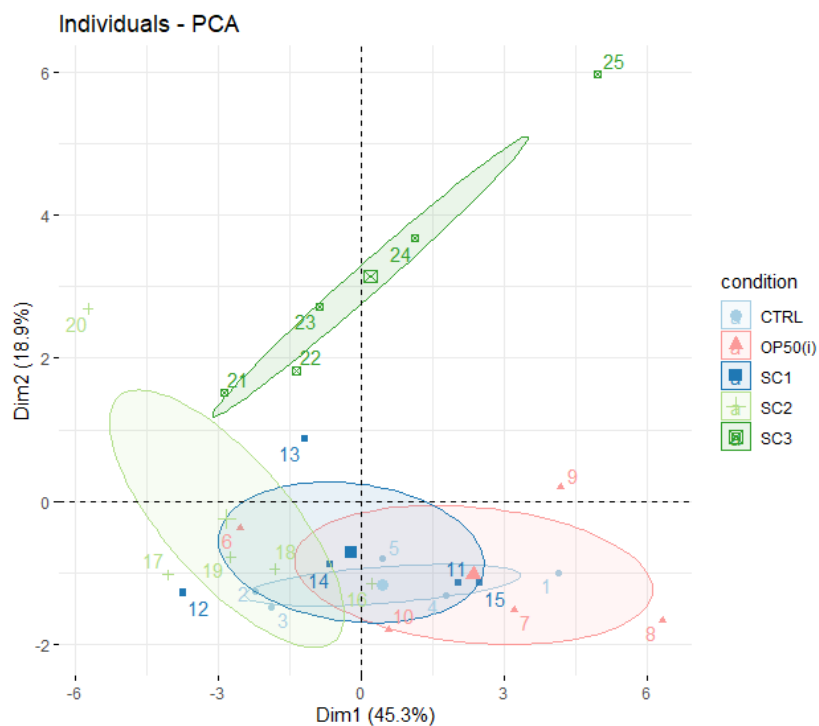


Figure S2. Graph of individuals of the Principal Component Analysis on FAMES (5 point for each condition corresponding to each replicate (CTRL 1-5, OP50(i) 6-10, SC1 11-15, SC2 16-20, SC3 21-25 and confidence ellipses at 95% for each condition)

| FAMES | Correlation with dose | Dim.1 (%) | Dim.2 (%) |
|-------------|-----------------------|-----------|-----------|
| C12:0 | 0.50* | 5,20 | 6,14 |
| C14:0 | 0.29 | 7,36 | 4,73 |
| C15:0 | 0.07 | 8,57 | 0,16 |
| C16:0 | -0.17 | 5,15 | 0,00 |
| C16:1(9) | 0.53* | 0,78 | 22,83 |
| C16:1 | 0.59** | 0,18 | 8,33 |
| C17:0iso | 0.05 | 6,62 | 0,89 |
| C17:0 | 0.26 | 5,52 | 5,52 |
| C17Δ | -0.73** | 5,39 | 10,16 |
| C18:0 | 0.03 | 7,54 | 1,33 |
| C18:1(9) | -0.09 | 7,93 | 0,01 |
| C18:1(11) | -0.29 | 9,28 | 0,87 |
| C18:2(9-12) | -0.39 | 9,35 | 1,75 |

| | | | |
|-----------------|---------|------|-------|
| C18:3 | 0.28 | 0,10 | 8,08 |
| C19:0 | -0.50* | 0,97 | 0,12 |
| C18 (3-hydroxy) | -0.60** | 6,45 | 3,25 |
| C19 Δ | -0.64** | 4,23 | 12,90 |
| C20:3 | -0.18 | 0,94 | 0,74 |
| C21:5 | -0.36 | 3,93 | 7,05 |
| C22:1 | 0.29 | 4,55 | 5,13 |

Table S2. Spearman correlation between total dose (see Table S1) and FAMES content (**' p<0,05;'**' p<0,01) and 'FAMES' variable contribution to PCA axis

Assessment of effects on food behavior and assimilation

Bacterial lawn avoidance assay

At the end of exposure, L4/YA worms from controls, OP50(i) and SC3 were transferred into fresh NGM plates with un-irradiated OP50 lawn. 20 worms were individually deposited onto the NGM plates in triplicates (for a total of 60 worms per condition), in an equidistant manner outside of the bacterial lawn. The number of worm reaching the bacterial lawn was measured during 28h, every hour during the first 8h and from 24 to 28h. Percentage of occupancy was calculated by dividing the number of worms in the bacterial lawn by the total number of worms deposited.

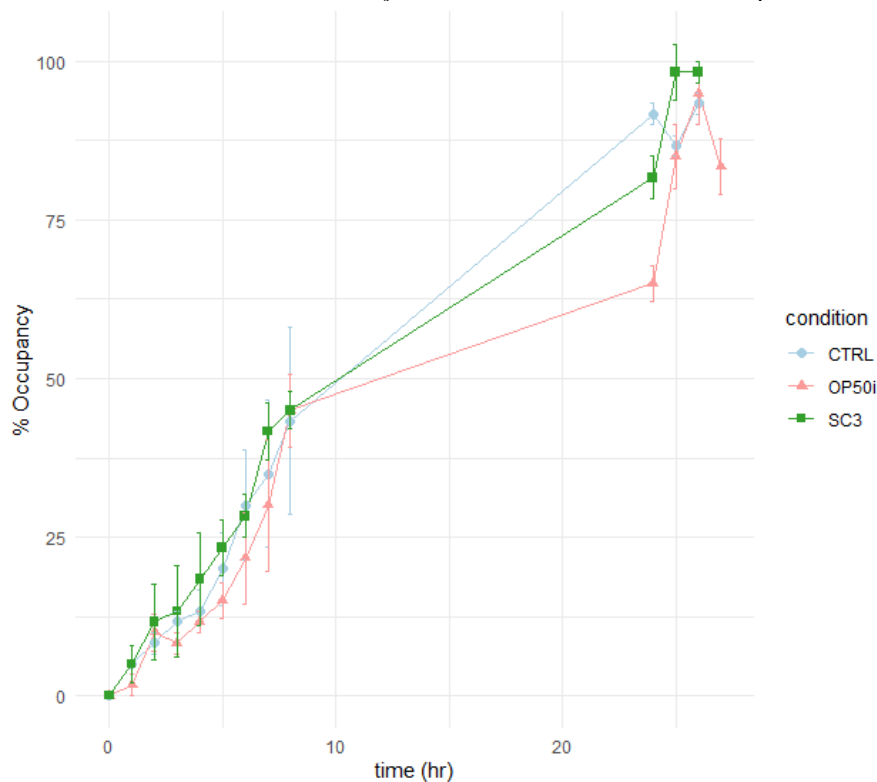


Figure S3. Time course of attraction of nematodes to *E. coli* OP50 lawn for each condition. Error bars indicate SEM.

Thrashing assay

At the end of exposure, ~20 L4/YA worms per condition were deposited into 12-well NGM plates. Worms were left for a few minutes to get rid of bacteria, then 1mL of M9 was added to the plate onto the worm. Worms were left ~30 seconds to adjust to the liquid medium, then the number of thrashes defined by a single inflection back and forth was counted for 30s.

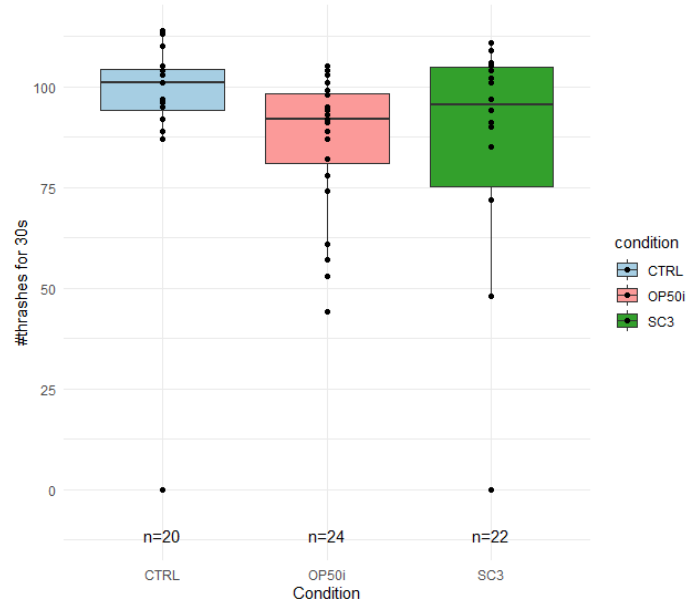


Figure S4. Number of thrashes for 30s for each condition (Kruskal-Wallis)

Assimilation of OP50

At the end of exposure, worms were rinsed and transferred into NGM plates containing OP50 previously stained with cyanine 5 (Cy5). Worms were left to feed onto the stained bacteria for ~6h then lightly rinsed and mounted onto slides with agar pad and a drop of 25mM NaN3. Images were obtained with a LSM 780 confocal microscope (Zeiss) using a 20x dry at a resolution of 12 bits, and the red HeNe laser (633 nm) for excitation of Cy5. Whole worm intensity and intensity only inside the digestive tract were measured using the ROI and measure tools in FIJI. Assimilation efficiency was then calculated using the formula explained in Supplementary Data (Figure S6).

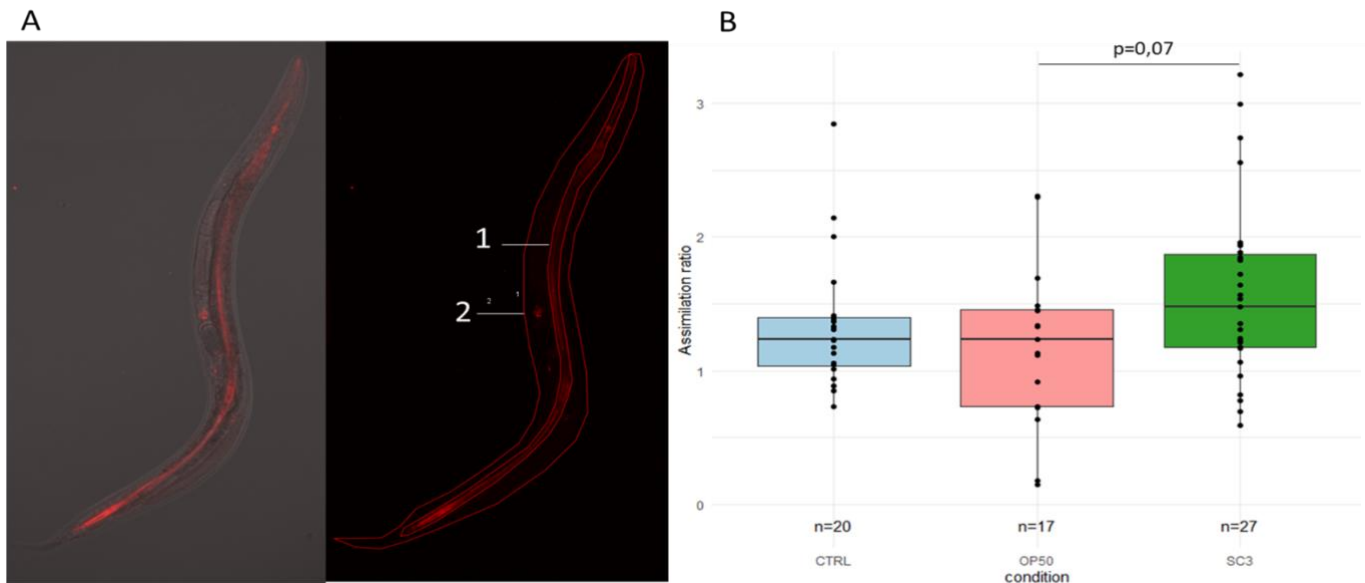


Figure S5. (A) Method for measurement of assimilation rate (Cy5-stained OP50 in the intestine). (B) Assimilation ratio* for each condition (Anova, Tukey post-hoc)

$$* \text{ Assimilation ratio} = \frac{(\text{Intensity 2} - \text{Intensity 1})}{\text{Intensity 1}}$$

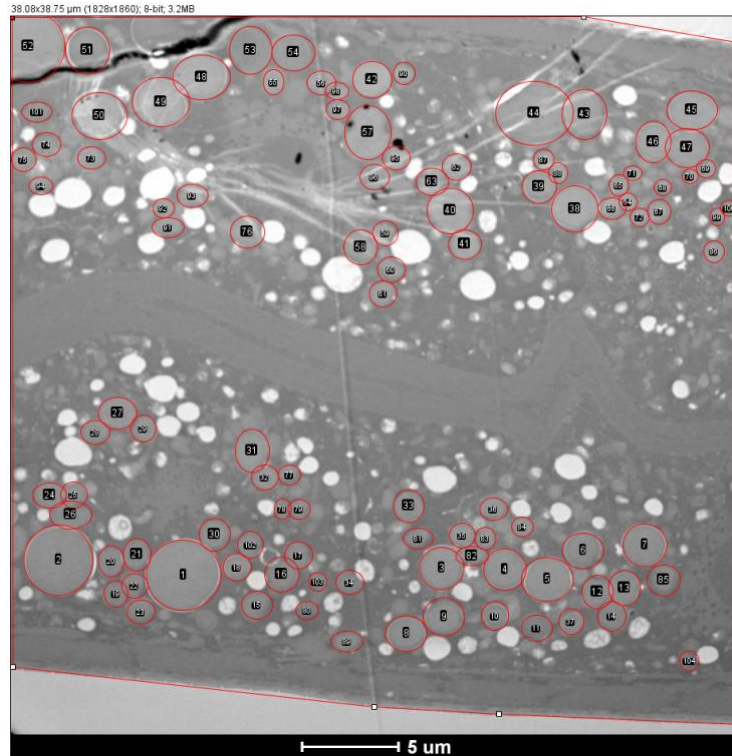


Figure S6. Example of analyzed image (CTRL) for lipid droplet area measurement using FIJI (ROI Manager tool)

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PAPER II

Developmental pathways involved in the broodsize decrease in *Caenorhabditis elegans* exposed to ionizing radiations

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1. Introduction

Chronic exposure to ionizing radiations induce several defects in organisms, through a variety of effects ranging from direct ionizations of molecules (DNA, lipids, proteins, etc.) to indirect effects such as oxidative stress via the production of reactive oxygen species (ROS) [1,2]. Cells exhibit many types of repair mechanisms and defence systems to counteract these damages [3,4]. However, when repair mechanisms and defence systems are overwhelmed, accumulation of ROS or direct damages in cells can result in the alteration of biological pathways leading to different cell fates such as mutations, cell cycle arrest or apoptosis as well [5]. Repair mechanisms, and thus cell fate, differ according to the cell type (e.g., somatic, germinal) and progression into the cell cycle (e.g., mitotic, post-mitotic) [6-10]. Accordingly, consequences at the individual level depend on the life stage exposed (e.g., gametogenesis, embryonic or post embryonic development...etc.) [11,12].

These primary processes can lead to adverse effects at larger scales i.e., individual and population. In particular, reproduction, which directly impacts population dynamics, is the most radiosensitive individual parameter in many invertebrates, including *Caenorhabditis elegans* [13-17]. However, the early molecular events of this reproduction decrease are still not all elucidated. Previous results showed a decrease of broodsize after chronic irradiation to gamma rays during whole development of *C. elegans* (upon 50 mGy.h⁻¹), concomitant with a decrease in sperm number and egg-laying rate [18-20]. In particular, we observed that 90% of the broodsize decrease can be attributed to sperm decrease, and 10% to others, yet unexplained, phenomena, probably linked to ovulation process [18].

Despite these elements, knowledge gaps remain regarding the underlying mechanisms contributing to these processes. Knowledge of the development of *C. elegans* germline is crucial to understand the timing of occurrence of these pathways. In hermaphroditic *C. elegans*, spermatogenesis and oogenesis are temporally separated events [21]. Spermatogenesis begins at the late L3 stage, prior to oogenesis, to produce a stock of ~300 spermatozoa (~150 per gonad arm stored in each spermatheca) [22]. Oogenesis then takes

place constitutively from the late L4 stage until the late life of the nematode, during which many female germ cells undergo the apoptotic program (namely, physiological apoptosis). This transition from spermatogenesis to oogenesis is regulated by the sex determination module involving many regulators [23-25].

As sperm count is finite unlike oogenesis, we hypothesized that a disruption of the spermatogenesis/oogenesis transition, via TRA-1 or MPK-1 could induce the observed decrease in sperm count. Indeed, among the several germline regulatory pathways, the Ras/ERK (MPK-1) controls developmental processes in *C. elegans* [30]. In particular, this kinase protein is involved in the regulation of the spermatogenesis/oogenesis transition and thus the regulation of sperm count [26,27]. In addition, it is also involved in stress response pathways, especially after irradiation [28,29]. In *C. elegans*, MPK-1 expression is spatially and temporally dynamic, allowing us to distinguish the different biological processes it regulates according to its zone of expression in the gonad [30]. Notably, in the pachytene zone, MPK-1 regulates the mitosis/meiosis transition and the spermatogenesis/oogenesis transition, while in developing oocytes it regulates oocyte maturation and ovulation. Sex determination is notably regulated by *tra-1* [23,25]. TRA-1/GLI is the master regulator of sex determination in nematodes and acts by promoting female fate [31,32]. In addition, it is also involved in several stress pathways such as the regulation of apoptosis via *egl-1* [33,34] and *daf-16*/FOXO, a major target of the insulin/IGF-1 signaling (IIS) regulating aging and oxidative stress response [35].

Besides, to validate that in chronic, in male germ cells, neither DNA-damage induced, nor physiological apoptosis occurs [36-38] but only during oogenesis in female germ cells, apoptosis was also assessed. In response to DNA damages, the apoptotic pathway is triggered by other regulators than physiological apoptosis (i.e., normal apoptosis occurring during oogenesis) [36]. In both cases, the downstream core apoptotic pathway remains the same. CED-3 is the final caspase protein of the core apoptotic pathway in all cell types in *C. elegans* [39]. In germ cells, apoptosis is restricted to late pachytene stage germ cells at the L4 stage [36].

Thus, in this study we tested potential pathways that are known to be involved in both stress response and developmental processes in the *C. elegans* germline. In regard to these processes, nematodes were chronically irradiated at different life-stages as previously done (e.g., embryogenesis, early gonadogenesis, whole development) [18]. Then we investigated the spermatogenesis/oogenesis switch via TRA-1 and MPK-1 activity, and the core apoptotic pathway via CED-3 to explain the observed decrease of sperm number and ovulation rate upon exposure to chronic ionizing radiations at different life stages.

2. Material and methods

Strains and maintenance

The following strains were provided by the *Caenorhabditis* Genetics Centre (University of Minnesota, St. Paul, MN, USA): Bristol N2 strain was used as wild type, MT1522 (*ced-3(n717)*) was used for the analysis of apoptotic pathway, DZ840 (TRA-1::GFP) was used for the analysis of TRA-1 expression. Nematodes were maintained as described previously [18].

Irradiation design

Worms were synchronized and irradiated in the MIRE facility at the IRSN laboratory (Cadarache, France), as previously described in [18]. Wildtype worms (later stained with DAPI and MPK-1 antibody) were irradiated according to the previous three scenarii of exposure (SC1 – *in utero* until embryogenesis; SC2 – *in utero* until the beginning of gametogenesis; SC3 – *in utero* until the end of development – refer to [18] for details of scenarii). MT1522 were irradiated during whole development only (i.e., SC3) while DZ840 were irradiated during whole development (i.e., SC3) and until the beginning of gametogenesis (i.e., SC2).

Sample preparation

Following exposure at different scenarii, wildtype N2 worms were all collected at the L4-YA (Young Adult) stage, rinsed in M9 and quickly dissected and treated for dpMPK-1 (Sigma) staining according to Gervaise and Arur, 2016 protocol [30]. Transgene DZ840 worms were collected and washed in M9, mounted *in vivo* on 3% agar pads on slices with a drop of 100mM NaN₃ and images were directly acquired. For MT1522, ~20 worms per condition (CTRL vs SC3) were picked in individual petri dishes for reproduction assay, while the rest were rinsed in M9 and treated for DAPI staining and sperm counting as previously described [18].

Image acquisition and intensity quantification

For dpMPK-1-stained wildtype worms, images were collected on a Zeiss LSM780 confocal microscope (Carl Zeiss, <https://www.zeiss.com/>) (Zoom platform of the CEA – Cadarache, France), using a 40x objective (Plan Apo water N.A. 1,2). Images were acquired in 12 bits using Zen black software (SP2 v.11.0, 2012, Carl Zeiss). Alexa Fluor 555 conjugated to anti-mouse secondary antibodies (Invitrogen, Waltham, MA, USA) was excited with a 561nm Diode-Pumped Solid State (DPSS) Lasers and emitted light was collected from 564 to 573 nm using the MBS 561 filter. DAPI was excited with a 405nm Lasers and emitted light was collected from 450 to 495 nm using the MBS 405 filter. The measured intensity at different areas (zone 1: 80µm, and zone 2: -1 oocyte – see Figure 1) was weighted by the intensity at the loop level (0µm) where the signal remains stable whatever the condition. The intensity is thus reduced to a ratio of the intensity at 80µm/0µm for zone 1, and -1 oocyte/0µm for zone 2.

For DZ480 and MT1522 worms, images were acquired using ZEISS apotome as previously described [18]. In MT1522, sperm number was manually counted using the ImageJ/FIJI software [40]. In DZ480 strain, the intensity was measured using Histolab software v.12.0.1 (Advilab laboratory). We investigated (1) total intensity in the worm and (2) the intensity in hypodermis and muscle cells expressing TRA-1 as described by Bayer et al, 2020 [41].

Antibodies and reagents

The following antibodies and reagents were used in this study: Sigma monoclonal anti-activated MAP kinase (diphosphorylated ERK-1&2) antibody (M8159), Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 555 (Invitrogen, Waltham, MA, USA), Vectashield antifade mounting medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA).

Statistical analysis

All data were obtained with a simple random sampling. Count data (spermatids count, total broodsize, hatching success, egg laying rate) were analyzed using a GLM (General Linear Model). For statistical significance of intensity, data was log transformed to follow a normal distribution, means were compared using an Anova. For each test, post-hoc analysis was conducted (Dunnett for parametric tests) and p-values were adjusted using the Bonferroni correction. An alpha risk of 5% was taken as significant. Statistical analysis was conducted on R Studio software, Version 1.1.423 (© 2009–2018 RStudio, Inc., Boston, MA USA), using the following packages for statistical tests and data visualization: 'ggplot2', 'ggpubr', 'dplyr', 'Hmisc', 'tibble', 'tidyr', 'ggthemes', 'car', 'EnvStats', 'multcomp', 'dunn.test', 'fitdistrplus', 'rstatix'.

3. Results

We investigated the involvement of three molecular pathways in the reprotoxic response to chronic ionizing radiations exposure in *C. elegans*: Ras/ERK (MPK-1) pathway, apoptotic pathway through CED-3, and sex determination pathway through TRA-1.

3.1 MPK-1 is activated in the pachytene zone of the germline upon chronic exposure to IR

Figure 1 show the expression dynamic of dpMPK-1 in *C. elegans* L4/YA (young adult) hermaphrodite germline and the zones of interest that were investigated.

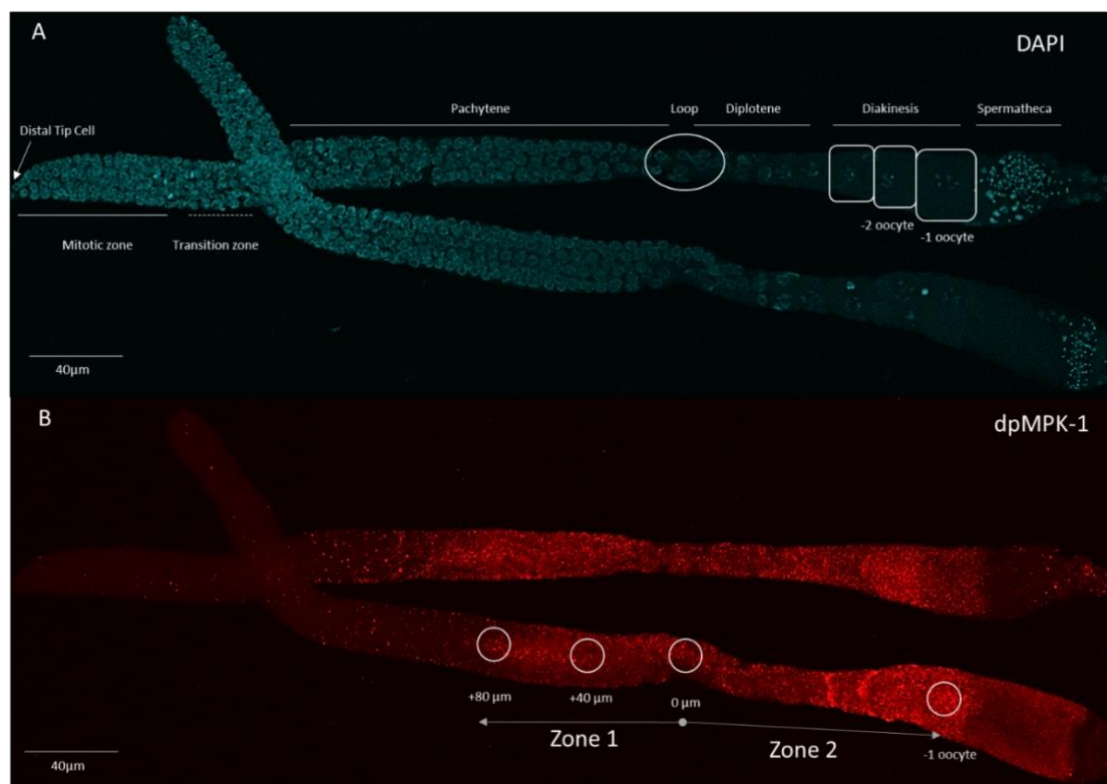


Figure 1 (A) DAPI-stained L4/YA hermaphrodite *C. elegans* germline. Germ cells differentiate from the distal end of the gonad (distal tip cell on the left) to the proximal end (spermatheca on the right). (B) dpMPK-1

expression in *C. elegans* dissected gonad (Zone 1 corresponds to expression in the pachytene stage, 2 measures of intensity were taken at 80 μ m and 40 μ m from the loop (0 μ m); Zone 2 corresponds to expression in the proximal oocytes).

Figure 2 shows the intensity of dpMPK-1 expression in zone 1 and zone 2 as defined in Figure 1. In zone 2, where dpMPK-1 controls the oocyte-linked processes (maturation, ovulation), no significant difference between irradiated conditions and control was found. On the other hand, in zone 1, while the measurement of intensity at 40 μ m from the loop showed no significant difference between conditions (Figure S1), dpMPK-1 is significantly over expressed at 80 μ m from the loop after irradiation throughout development (SC3) and after irradiation throughout embryogenesis (SC1). This can suggest an earlier activation of MPK-1 in the germline after irradiation.

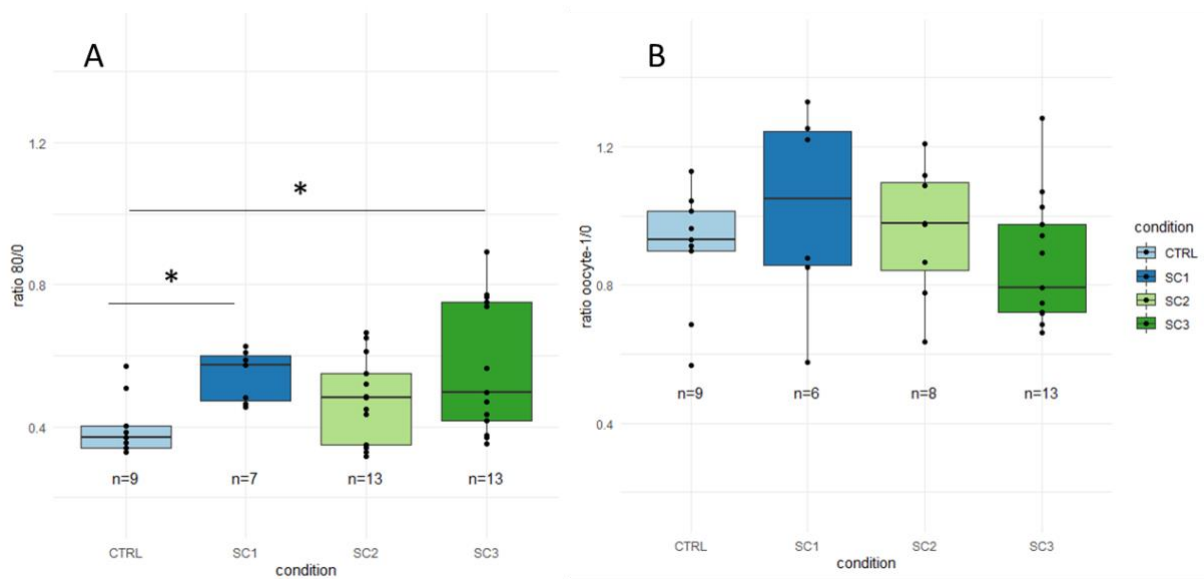


Figure 2 (A) dpMPK-1 intensity in Zone 1 (intensity at 80 μ m weighted by intensity at the reference area 0 μ m) for each condition (SC1: *in utero* until embryogenesis; SC2: *in utero* until the beginning of gametogenesis; SC3: *in utero* until L4/YA – whole development) (B) dpMPK-1 intensity in Zone 2 (intensity at oocyte -1 weighted by intensity at the reference area 0 μ m) for each condition (Anova, Dunnett post hoc with Bonferroni adjustment, '' p value < 0.05)**

Since MPK-1 did not seem to be involved in the regulation of sperm count, we continued to investigate the sex determination pathway via TRA-1.

3.2 Expression of TRA-1 (master regulator of sexual determination) is unchanged in response to radiations

No expression was observed in the germline and many gut granules autofluorescence can be observed (Figure 3A). Moreover, we noted an important autofluorescence of non-transgenic worms with images acquired under the same conditions (Figure S2). Transgenic worms showed a global intensity only 1.2 times higher than wildtype N2 worms, and standard deviation was important (Figure S3). Nonetheless, concordant with the literature, we observed a global stronger expression in L4-YA worms than in L3 worms [41].

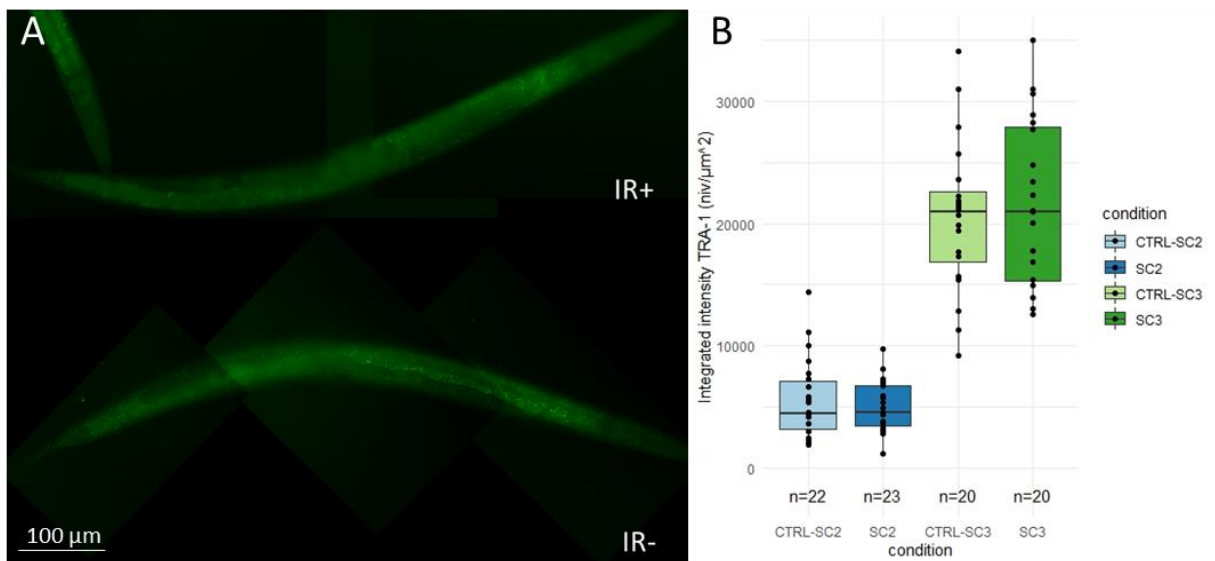


Figure 3 (A) TRA-1::GFP expression in L4/YA hermaphrodite *C. elegans* (IR+ : irradiated worms during whole development (SC3), IR- : control worms) **(B)** TRA-1 intensity after exposure at different scenarii and associated controls (SC2 : *in utero* until the beginning of gametogenesis, SC3 : *in utero* until L4/YA stage)

To alleviate this autofluorescence, another more selective method for measuring intensity was used as follow.

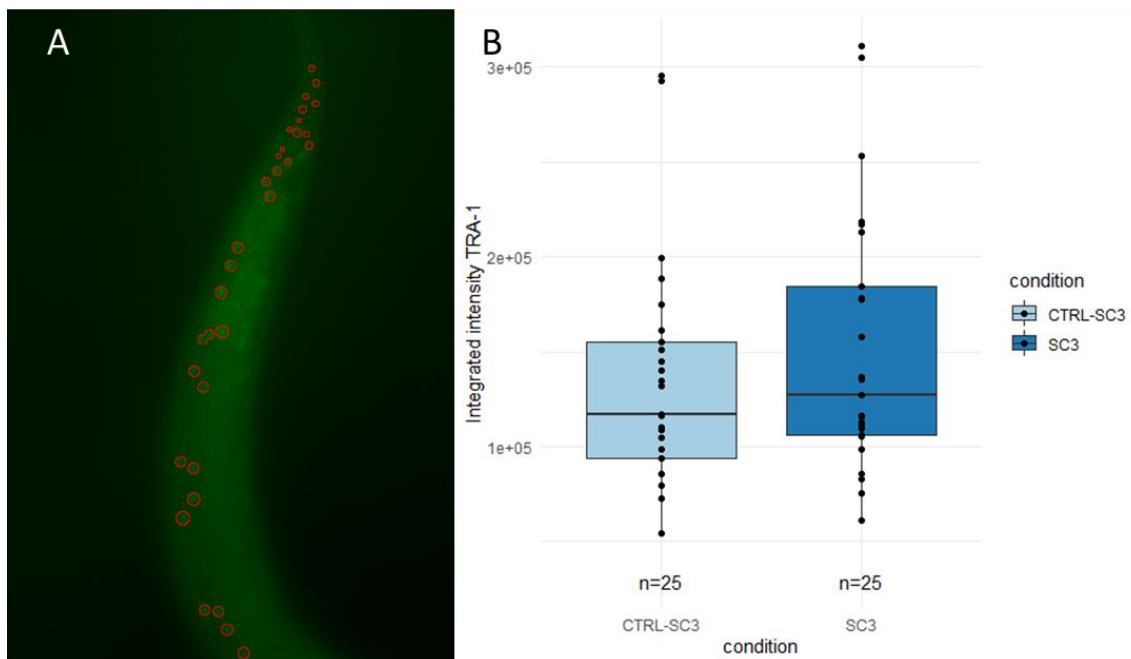


Figure 4 (A) Selective measurement of TRA-1::GFP intensity in hypodermis and muscle cells **(B)** Intensity in hypodermis and muscle cells in worms irradiated throughout whole development (SC3).

No difference was observed between the conditions, regardless of the method of measurement (Figure 3 & Figure 4).

Considering that TRA-1 did not seem to be involved in the reduction of sperm count upon chronic exposure to IR, we then investigated the implications of the apoptotic pathway in response to IR.

3.3 Apoptotic pathway induces ovulation rate decrease but no effect on sperm upon chronic exposure to IR

Figure 5 shows no effect of radiations on egg-laying rate in irradiated *ced-3(n717)* worms (Figure 5A), contrarily to what was previously observed in N2 (Figure 5B results from [18]) showing a decrease of 35%, p-value < 0.001).

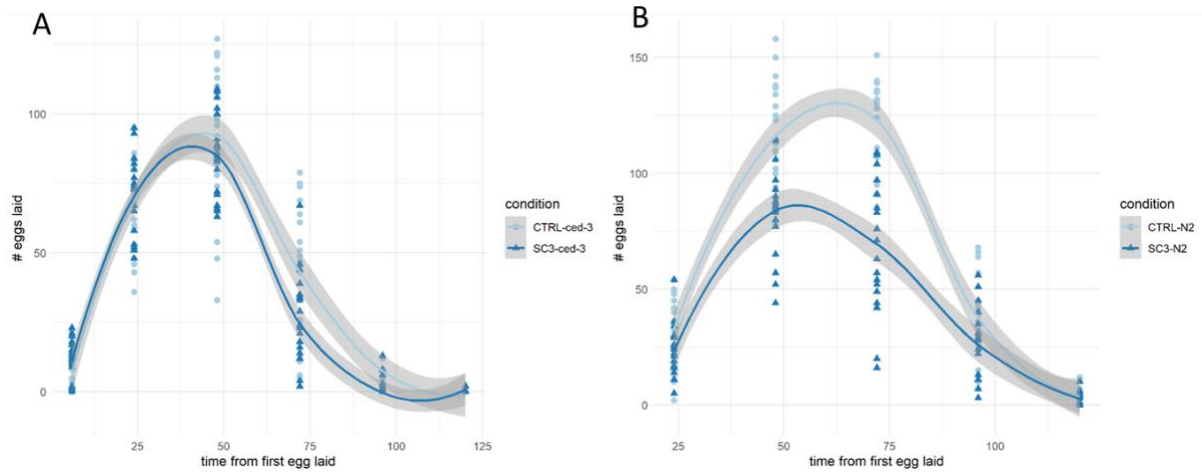


Figure 5 (A) *ced-3(n717)* egg-laying rate for each condition (CTRL, SC3: irradiated during whole development; GLM, Dunnett post-hoc with Bonferroni adjustment, p-value = 0.74) (B) N2 egg-laying rate for each condition (GLM, Dunnett post-hoc with Bonferroni adjustment, p-value < 0.01)

Figure 6A shows a slight broodsize decrease in irradiated *ced-3(n717)* worms compared to controls (-15%; p-value = 0.02). A higher decrease was previously observed in N2 between irradiated worms and controls (-35%; p-value < 0.001) (Figure 6B results from [18]).

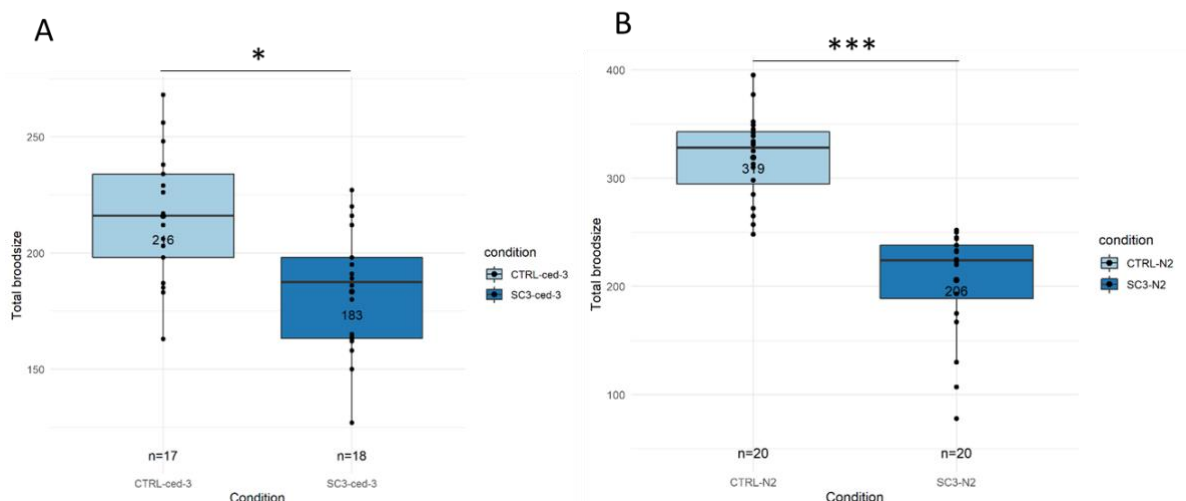


Figure 6 (A) *ced-3(n717)* total broodsize for each condition (B) N2 total broodsize for each condition (CTRL, SC3: irradiated during whole development; GLM, Dunnett post-hoc with Bonferroni adjustment; '*' p value < 0.05, '*' p value < 0.01)**

Figure 7A shows a decrease in sperm number in irradiated *ced-3(n717)* worms compared to controls (-15%; p-value < 0.01) equivalent to the *ced-3(n717)* broodsize decrease observed in Figure 6. A similar decrease in sperm number was previously observed in N2 under the same conditions of irradiation (Figure 7B) (-23%; p-value < 0.001) [18].

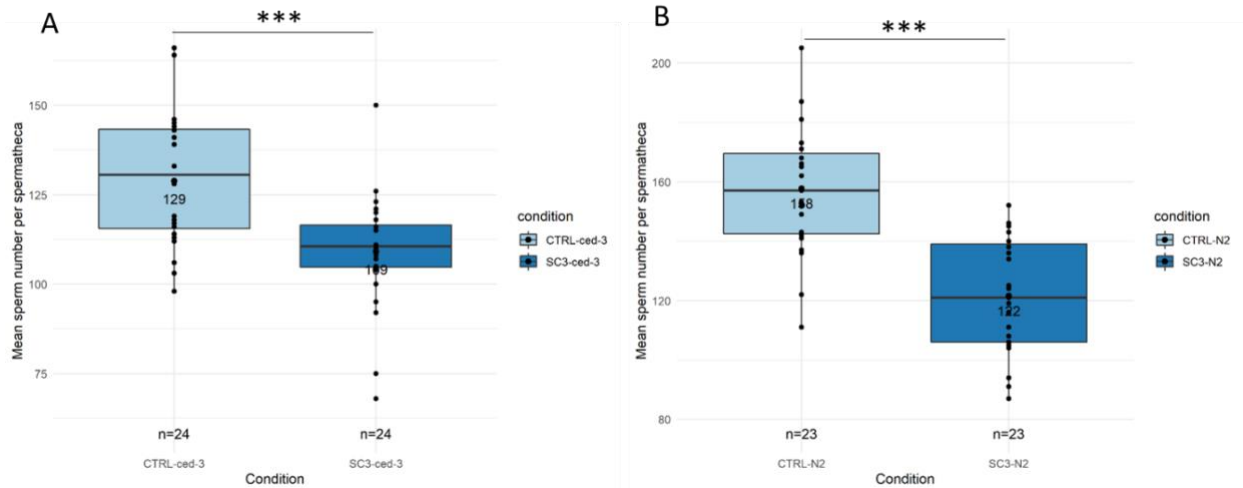


Figure 7 (A) *ced-3(n717)* sperm number for each condition (B) N2 sperm number for each condition (CTRL, SC3: irradiated during whole development; GLM, Dunnett pot-hoc with Bonferroni adjustment, ‘*’ p-value < 0.01)**

4. Discussion

Our first hypothesis to explain the cause of sperm number decrease was through the sex determination module. The transition from spermatogenesis to oogenesis is controlled by several processes including protein myristoylation, in which the myristic acid (C14) plays a central role [42]. In our previous lipid analysis [18], no significant effect was found on C14, which discarded this hypothesis. Another important regulator of the sex determination module is the Ras/ERK (MPK-1) pathway. This pathway controls many developmental processes in the germline, such as progression into meiosis [43-46] and continued proliferation [47-49], germ cell apoptosis [36], and oocyte maturation [46,50]. In particular, it promotes spermatogenesis and spermiogenesis [26,51]. Moreover, MPK-1 is involved in stress response [28,29,52], in particular radio-induced apoptosis [36,53]. Our results show an overexpression of dpMPK-1 upon exposure throughout the whole development in the pachytene zone (i.e., zone 1), where it controls the progression into meiosis and apoptosis. This result is concordant with the literature where an activation of MPK-1 upon acute IR exposure was found [28], and upon chronic IR, an upregulation of a target of MPK-1 (*toe-4*) was measured [20]. Since MPK-1 is sperm-promoting, its overexpression here cannot be linked to the observed sperm decrease. However, it is probably linked to the increase of radio-induced apoptosis. In mammals, activation of the ERK1/2 pathway occurs in cancer cells by inducing proliferation upon DNA damage and other external stimuli [54,55]. In *C. elegans*, recent findings show that an isoform of MPK-1 acts non-autonomously to promote germ stem cell (GSC) proliferation from the gut or somatic gonad [49]. Only the isoform acting autonomously in the germline was analysed in our study, therefore we cannot conclude precisely on MPK-1 activity regarding GSC proliferation in response to IR exposure.

We then focused on TRA-1 which is the master regulator of sex determination in *C. elegans* [23,25]. It promotes female fate [31,32] by preventing the expression of male genes like *fog-3* in germ cells [56], *egl-1* in the HSN neurons [34], and *mab-3* in the intestine and tail [57], thus promoting vitellogenin expression [58,59]. Previous results under the same conditions of exposure showed an overexpression of vitellogenins and *egl-1*. Moreover, TRA-1 directly regulates *daf-16*, a target of the IGF-1/Insulin like signalling pathway (IIS) [35] which was previously shown to be involved in response to IR [19]. We thus hypothesized that *tra-1* could be involved in the response to IR in our conditions. However, our results showed no difference in TRA-1 expression between irradiated worms and controls. It then seems that regulation of *vit* genes and *egl-1* are independent from TRA-1 expression in the hermaphrodite in response to IR, as well as the regulation of sperm number.

Considering that the sex determination pathway did not seem to be involved in sperm decrease, our next hypothesis was linked to apoptosis. Previous studies had shown the implication of apoptosis in the nematode *C. elegans* upon chronic exposure to ionizing radiations (IR), via the expression of the proapoptotic gene *egl-1* [60] and increase of apoptotic cell corpses [20]. However, it was not clearly known to which extent it contributes to broodsize decrease. Our analysis of the core apoptotic pathway revealed that radio induced apoptosis is not involved in sperm decrease but is involved in ovulation rate decrease. Indeed, sperm number is decreased after irradiation in *ced-3(n717)* mutants in the same way as in N2 [18], which means that this decrease occurs via another mechanism than apoptosis. On the other hand, the ovulation rate is not affected by irradiation in *ced-3(n717)* mutants, contrary to what was observed in N2, which means apoptosis is involved in the decrease of the ovulation rate in N2 after chronic irradiation, probably even independently from the regulation by spermatozoa. Indeed, we previously hypothesized that the ovulation rate decrease was a direct consequence of sperm decrease since meiotic maturation of oocytes and ovulation are regulated by sperm-dependent signal via the Major Sperm Protein (MSP) [27,61]. Overall, broodsize decrease in *ced-3(n717)* mutants can be 100% attributed to sperm decrease, which confirms that the decrease of the broodsize in N2 is not only due to the sperm number but also to the decrease of ovulation, through the apoptotic pathway. Previous studies showed that lipids and proteins involved in oogenesis processes were misregulated upon exposure to IR, e.g., vitellogenins, transporters of yolk granules necessary for oocyte maturation [18,62]. Concordant with our present finding, it seems that radio-induced apoptosis in oocytes might be an initiating event of this protein and lipid misregulation.

From this study, we show that 10% of *C. elegans* broodsize decrease can be attributed to radio-induced apoptosis in oocytes, while 90% of this decrease is attributable to sperm decrease. Figure 8 illustrates the proposed model for mechanisms occurring upon chronic exposure to IR in the nematode *C. elegans* and contributing to broodsize decrease.

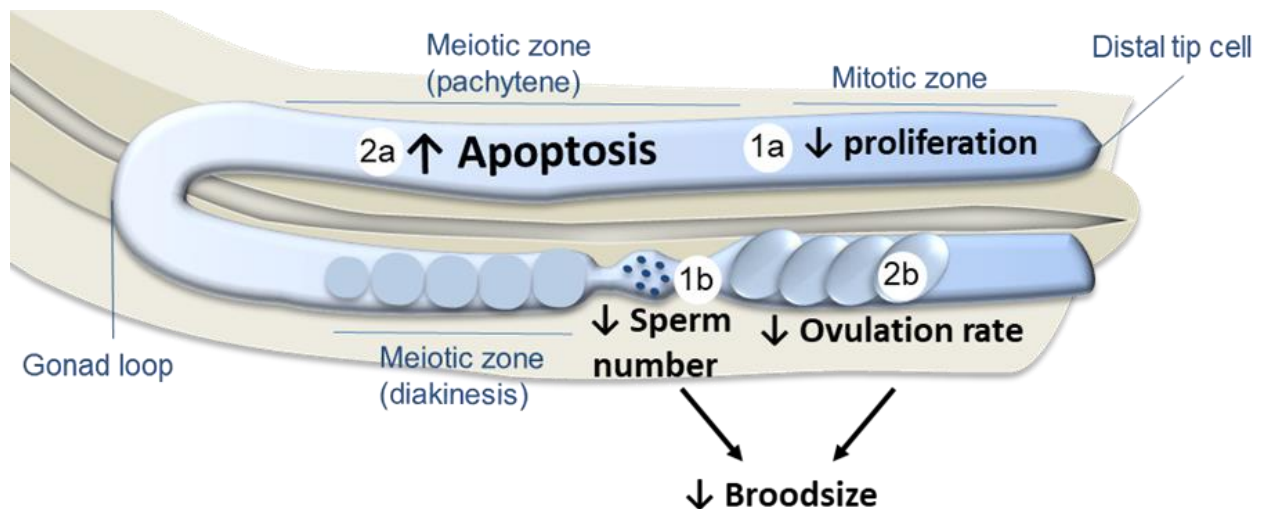


Figure 8 Proposed conceptual model for mechanisms causing broodsize decrease in hermaphrodite *C. elegans* exposed to chronic ionizing radiations. Two mechanisms (1 and 2) act in parallel that affect oogenesis and spermatogenesis independently and reduce broodsize. The decrease of cell proliferation in the mitotic zone (1a) was previously observed through cell cycle arrest [19,60] and the decrease of the size of the mitotic zone [19]. This decrease could directly lead to a decrease in sperm number (1b). In parallel, ionizing radiations induce an increase of apoptosis in germ cells (this study, [20,60], which directly leads to a decrease of ovulation rate (this study).

We suggest that two mechanisms act in parallel that affect spermatogenesis and oogenesis independently both contributing to broodsize decrease. The causal mechanisms of sperm decrease remain unknown, but we show that neither the Ras/ERK (MPK-1) pathway, apoptotic pathway nor TRA-1 are involved in this decrease. However, among the observed results, it is interesting to stress out that sperm count does not decrease in early exposure scenarios (SC1, SC2) or when irradiated just during gametogenesis ([20] - equivalent total dose). Only chronic irradiation throughout the whole development causes this decrease, suggesting that there is cumulative damage during development. One of the essential developmental processes that could be impacted throughout development and contribute to gametogenesis in *C. elegans* is cell proliferation. Indeed, a decrease in GSC proliferation could directly induce a decrease in the number of spermatozoa since they are in finite number. Concordant with this hypothesis, an arrest of the mitotic cell cycle in G2 was previously found after acute exposure to IR [36] and under similar conditions of this study (i.e., chronic whole development exposure) associated with a decrease of the mitotic zone in the gonad [19,63]. In addition, previous proteomic analysis after chronic exposure revealed a decrease in expression of proteins linked to the MCM complex involved in DNA replication [62]. Although defects in cell proliferation can potentially affect both oogenesis and spermatogenesis, our present results show that ovulation rate is not affected by another phenomenon than apoptosis upon exposure to chronic IR. This might be because oogenesis is constitutive during adulthood unlike spermatogenesis which only leads to a fixed number of spermatocytes.

5. Conclusion

Our study aimed to investigate the implication of some developmental and stress pathways in the reprotoxic response to chronic IR in the hermaphrodite nematode *C. elegans*. We showed effects in both oocytes and spermatocytes but to different extents. Indeed, oocytes seemed to be only affected by apoptosis which led to a decrease of ovulation rate. On the other hand, spermatocytes were not affected by apoptosis, but we still observed a reduction of sperm count that we attributed to a defect in proliferation in germ stem cells. We showed that the Ras/ERK (MPK-1) pathway could be involved in both radio-induced apoptosis and cell proliferation defects. Another hypothesis for reduction of sperm count was through the sex determination pathway but we did not find any evidence that this pathway was affected by IR. Complementary studies to better understand the mechanisms of sperm decrease would help to better understand the radiosensitivity of spermatozoa in *C. elegans* and potentially other androdioecious protandrous species.

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Supplementary Information

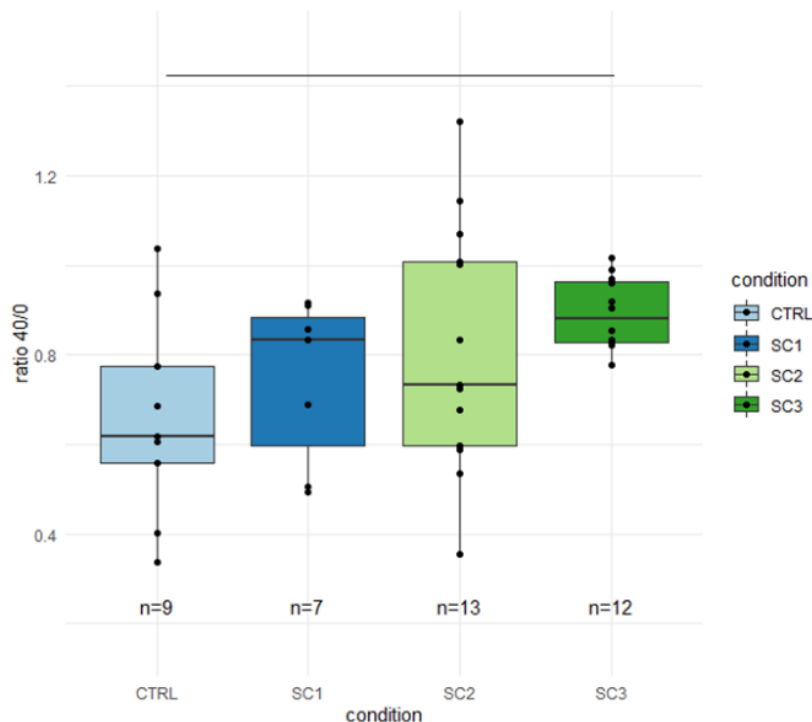


Figure S1 dpMPK-1 intensity in Zone 1 (intensity at 40 μ m weighted by intensity at the reference area 0 μ m) for each condition (SC1: in utero until embryogenesis; SC2: in utero until the beginning of gametogenesis; SC3: in

utero until L4/YA – whole development, Anova, Dunnett post hoc with Bonferroni adjustment, CTRL-SC3 p value = 0.058)

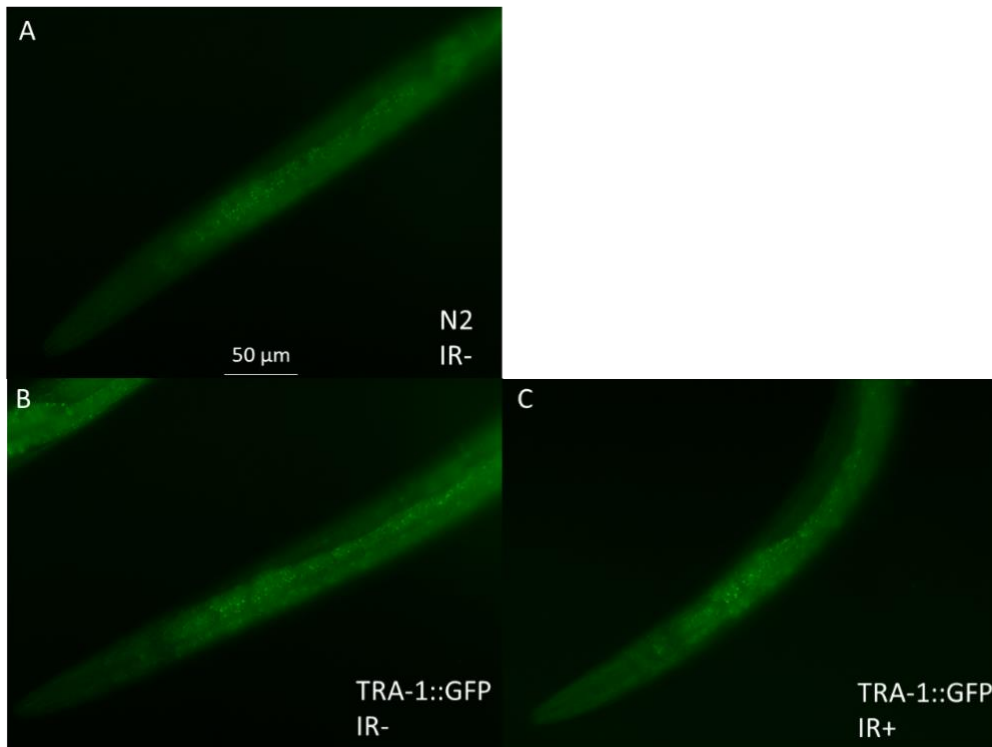


Figure S2 Autofluorescence of non-irradiated N2 worms (A) compared to non-irradiated transgenic DZ480 strain (TRA-1::GFP) (B) and irradiated transgenic DZ480 (TRA-1::GFP) strain (C). All worms are aged L4/YA stage.

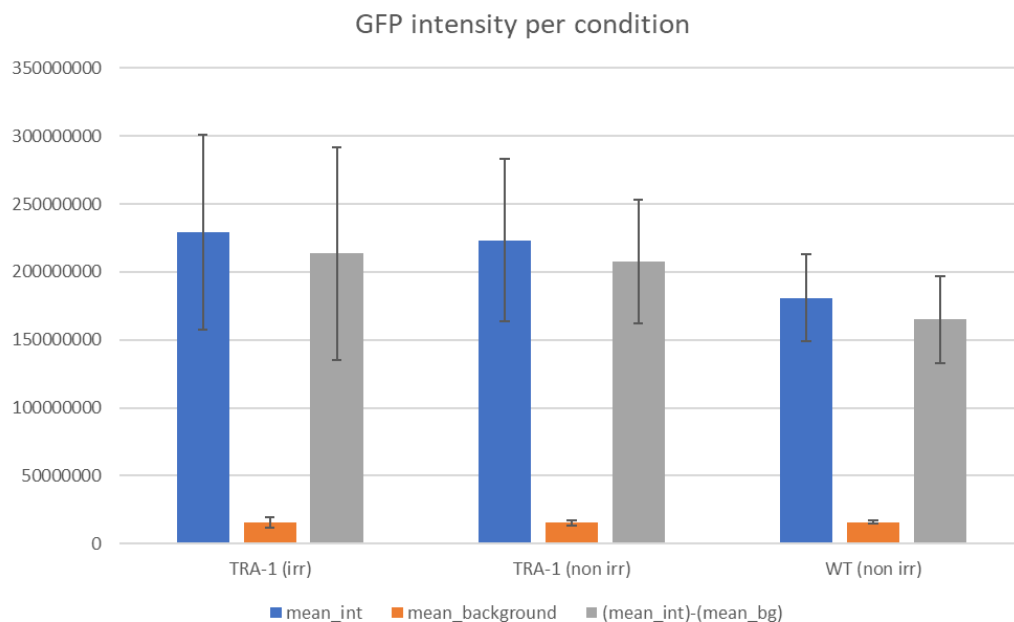


Figure S3 Total integrated intensity of WT worms (N2) vs transgenic DZ480 strain (irradiated and non-irradiated). Error bars are standard deviations.

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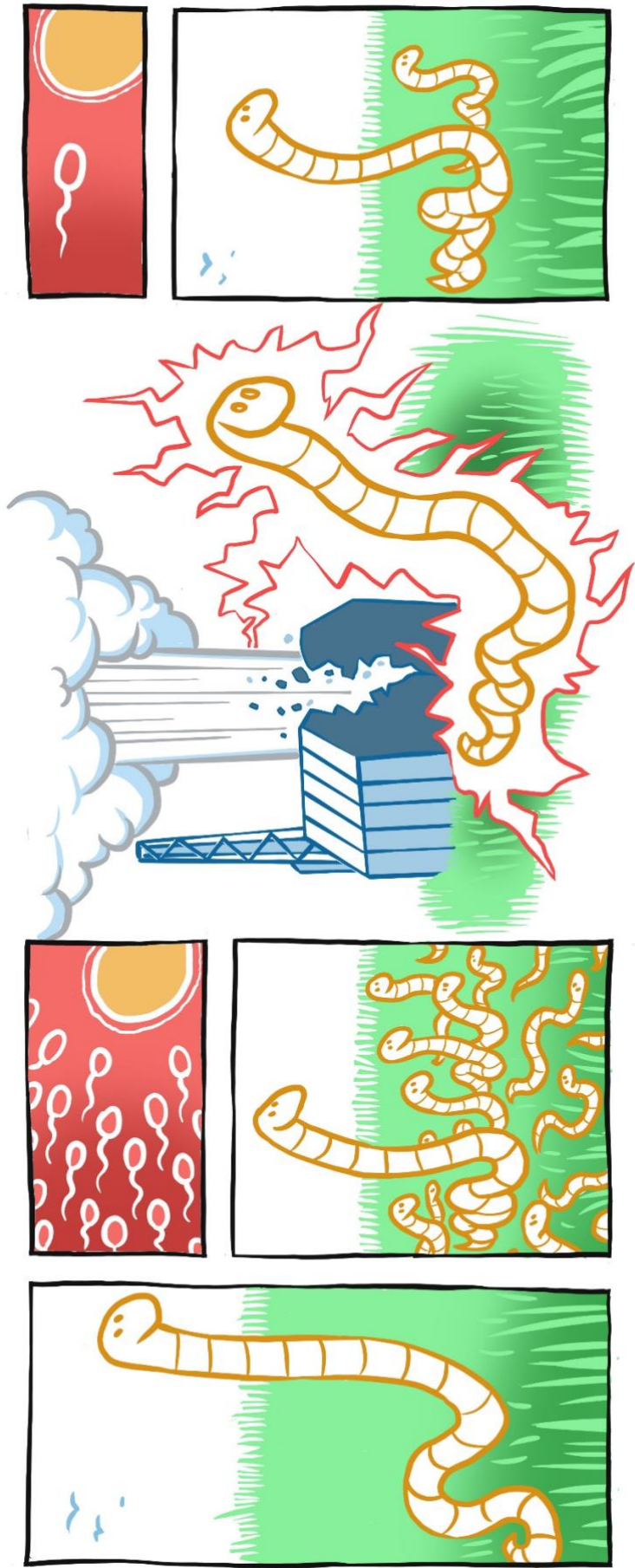
PUBLICATIONS AND COMMUNICATIONS

PUBLICATIONS

- Dufourcq-Sekatcheff, E.; Cuiné, S.; Li-Beisson, Y.; Quevarec, L.; Richaud, M.; Galas, S.; Frelon, S. **Deciphering Differential Life Stage Radioinduced Reproductive Decline in *Caenorhabditis elegans* through Lipid Analysis.** *Int. J. Mol. Sci.* **2021**, *22*, 10277. <https://doi.org/10.3390/ijms221910277>
- Dufourcq Sekatcheff, E., Godon C., Quevarec, L., Camillieri, V., Galas, S., Frelon, S. **Developmental pathways involved in reproduction decrease in *Caenorhabditis elegans* exposed to ionizing radiations** (*in prep*)
- Dufourcq-Sekatcheff, E., [...], Frelon, S., Armant, O. **Applying the AOP concept to radioecology: challenges and insights** (*in prep*)
- Tollefsen, K-E., [...] Dufourcq-Sekatcheff, E., [...] Frelon, S. **Adverse outcome pathways (AOPs) for radiation-induced reproductive effects in non-mammalian species: state of science and identification of a consensus AOP network.** *IJRB* (submitted)
- Quevarec, L., Reale, D., Dufourcq-Sekatcheff, E., Car, C., Armant, O., Dubourg, N., Adam-Guillermin, C., Bonzom, J-M. **Male frequency in *Caenorhabditis elegans* increases in response to chronic irradiation.** *Evolutionary Applications*, **2022** <https://onlinelibrary.wiley.com/doi/10.1111/eva.13420>
- Quevarec L., Bonzom J-M., Dufourcq-Sekatcheff E., Armant O., Adam-Guillermin C., Réale D. **Ionizing radiation affects the demography and the evolution of *Caenorhabditis elegans* populations** (*in prep*)

COMMUNICATIONS

- SETAC Europe 2021
- Thesis days IRSN & CBS2 (University of Montpellier) 2019 - 2021
- Minute Thesis IRSN 2021
- Energy Days (EDF/Air Liquide/Total/IRSN) 2019



IRSN illustration of this thesis project for the 3 Minutes Thesis event

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