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EFFETS DES MÉTAUX SUR LE SYSTÈME SÉROTONINE DE LA MOULE (*MYTILUS EDULIS*) ET DU TROPHOBLASTE PLACENTAIRE HUMAIN

Par

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« Quand c'est que les temps venont durs…
faudrait qu'un houme qu'a une terre à lui asseyit de traverser l'orage sans lâcher.
Faudrait qu'i' se disit que c'est une bourrasque et que ça passera. »

La Sagouine - Antonine Maillet

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RÉSUMÉ

Le système de la sérotonine est présent chez les mollusques et le placenta humain. Elle y joue, entre autres, un rôle important dans la reproduction. Chez les mollusques, la sérotonine régule la ponte et la différenciation sexuelle. Chez l'humain, la sérotonine placentaire et maternelle joue un rôle dans le développement du trophoblaste placentaire et dans le développement neuronal et cardiaque du fœtus. La moule bleue et le placenta sont de bons bioindicateurs de la contamination de leur environnement respectif. Ils offrent donc un potentiel pour l'étude des effets de la contamination environnementale sur le système sérotonine. Les contaminants environnementaux, tels que les métaux, peuvent altérer le système de la sérotonine. Cependant, très peu d'études se sont intéressées aux effets des métaux sur le système sérotonine de la moule bleue et du placenta humain. L'hypothèse de recherche de ce projet doctoral est que de faibles concentrations de Mn, Pb et Cd altèrent, de manière sexe-spécifique, le système sérotonine, chez la moule bleue et le trophoblaste placentaire humain. L'objectif général est d'étudier l'effet des métaux sur le système de la sérotonine dans les moules (*in vivo)* et le trophoblaste humain (*in vitro*), deux modèles non invasifs et accessibles. Les objectifs spécifiques sont de : **1)** Développer un protocole efficace de sexage des moules ; **2)** Déterminer l'effet d'une exposition à long terme du Mn, Pb et Cd sur l'expression du SERT, les concentrations de sérotonine et l'activité de la MAO dans le la moule bleue et si les effets observés varient selon le sexe ; **3)** Déterminer l'effet d'une exposition au Mn et Pb sur le système de la sérotonine du trophoblaste placentaire humain. Des *Mytilus edulis* et la lignée BeWo (modèle du trophoblaste villeux) ont été exposées à de faibles concentrations de Mn, Pb et Cd. Le placenta ainsi que le sang maternel et de cordon ont été recueillis d'une cohorte de femmes enceintes et les concentrations de métaux ont été déterminées. Les résultats montrent une modification du système sérotonine par une exposition à de faibles concentrations de Mn, Pb et Cd. Chez les moules bleues, une diminution de l'expression de la protéine SERT (Mn, Pb et Cd) et de l'activité de la MAO (Cd) est observée. Les moules femelles ont une activité de la MAO plus élevée que les mâles. Chez le placenta humain, les concentrations de 5-HT (Mn et Pb), la protéine SERT (Pb), l'activité de la protéine SERT (Mn et Pb) et l'activité de la MAO (Pb) diminuent. Une diminution de l'expression de l'ARNm codant pour le récepteur 5-HT_{2A} *(HTR2A)* est observée aux concentrations plus élevées de Mn (> 305 nM) et de Pb (> 100 nM) dans le sang maternel. Cette étude montre que de faibles concentrations de Mn et de Pb et de Cd perturbent le système 5-HT chez *Mytilus edulis* et le trophoblaste placentaire humain. Puisque la sérotonine est impliquée dans les mécanismes de reproduction chez la moule et dans les fonctions du placenta humain, les modifications observées dans le système de la sérotonine pourraient avoir des répercussions sur les populations de moules, sur la santé de la grossesse et sur le développement du fœtus. Des études supplémentaires sont nécessaires pour mieux comprendre comment les métaux modulent le système 5-HT et pour déterminer si c'est un biomarqueur d'effets sur la reproduction des moules et la grossesse humaine.

Mots clés: Cadmium, Manganèse, Plomb, Transporteur de la sérotonine (SERT), Monoamine-oxydase, Recepteur 2A de la sérotonine (5-HT_{2A}R), BeWo, Reproduction

ABSTRACT

The serotonin system is present in molluscs and human placenta. It plays, among others, an important role in reproduction. In molluscs, serotonin regulates spawning and sexual differentiation. In humans, placental and maternal serotonin plays a role in the development of the placental trophoblast and in neuronal and cardiac fetal development. Blue mussels and placenta are good bioindicators of contamination of their respective environment. They therefore offer potential for studying environmental contamination effects on the serotonin system. Environmental contaminants, such as metals, can affect the serotonin system. However, very few studies have examined the effects of metals on the serotonin system of the blue mussel and human placenta. The research hypothesis of this doctoral project is that low concentrations of Mn, Pb and Cd alter, in a sex-specific manner, the serotonin system in blue mussels and the human placental trophoblast. The general objective is to study the effect of metals on the serotonin system in mussels (*in vivo*) and the human trophoblast (*in vitro*), two non-invasive and accessible models. The specific objectives are to: **1)** Develop an effective protocol for sexing mussels; **2)** Determine the effect of long-term exposure to Mn, Pb and Cd on the SERT expression, serotonin concentrations and MAO activity in the blue mussel and whether the observed effects vary depending on the sex; **3)** Determine the effect of an exposure to Mn and Pb on the serotonin system of the human placental trophoblast. *Mytilus edulis* and the BeWo cell line (villous trophoblast model) were exposed to low concentrations of Mn, Pb and Cd. Placentas and maternal and cord blood were collected from a cohort of pregnant women and concentration metals were determined. Results show a serotonin system modification by exposure to low concentration of Mn, Pb and Cd. In blue mussels, a decrease in the SERT protein expression (Mn, Pb and Cd) and MAO activity (Cd) is observed. Female mussels have a higher MAO activity than males. In the human placenta, 5-HT concentrations (Mn and Pb), the SERT protein expression (Pb), the SERT protein activity (Mn and Pb) and the MAO activity (Pb) decreases. A decrease in the expression of mRNA encoding for the 5-HT_{2A} receptor (HTR2A) is observed at higher concentrations of Mn (> 305 nM) and Pb (> 100 nM) in maternal blood. This study shows that low concentrations of Mn, Pb and Cd disrupt the system 5-HT in *Mytilus edulis* and the human placental trophoblast. Since serotonin is involved in reproduction mechanisms of mussels and human placenta functions, serotonin system modifications observed may impact the mussel population, the pregnancy health and the fetus development. Further studies are needed to better understand how metals modulate the 5 HT system and whether it is an effect biomarker for mussels reproduction and human pregnancy.

Keywords: Cadmium, Manganese, Lead, Serotonin transporter (SERT), Monoamine oxidase (MAO), Serotonin 2A Receptor (5-HT2AR), BeWo, Reproduction.

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$[Ca^{2+}]_i$	Concentration de calcium intracellulaire
5-HIAA	Acide 5-hydroxyindol acétique
5-HT	Sérotonine ou 5-hydroxytryptamine
$5\text{-}HT_{2A}R$ ou *HTR2A*	Récepteur de la sérotonine 2A
5-HTP	5-hydroxytryptophane
AADC	L-amino décarboxylase
AANAT	Aralkylamine N-acétyltransférase
ADN	Acide désoxyribonucléique
ALDH	Aldéhyde déshydrogénase
AMPc	Adénosine monophosphate cyclique
ARNm	Acide ribonucléique messager
BeWo	Cellules de choriocarcinome humain
Ca	Calcium
Cd	Cadmium
CF	Capillaires foetaux
CG	Cellule géante
Cl	Chlore
COX	Cyclooxygénase
DOI	2,5-diméthoxy-4-indoamphytamine
evCTB	Cytotrophoblaste extravilleux
HIOMT	Hydroxyindole O-méthyltransférase
IDO	Indoleamine 2,3-dioxygénase
ISRS ou SSRI	Inhibiteurs sélectifs de la recapture de la sérotonine
K	Potassium
LOD	Limite de détection
LOQ	Limite de quantification
LPO	Peroxydation des lipides
L-trp	L-tryptophane
MAO	Monoamine-oxydase
MAP39	Male associated polypeptide
Mel	Mélatonine
Mn	Manganèse
MT	Métallothionéine
Na	Sodium
OCT	Transporteur de cations organiques
Pb	Plomb
PLC	Phospholipase C
PMTA	Transporteur de monoamine de la membrane plasmique
ROS	Espèces réactives de l'oxygène
S15	Fraction cytoplasmique
SERT ou *SCL6A4*	Transporteur de la sérotonine

STB	Syncytiotrophoblaste
TDO	Tryptophane dioxygénase
TPH	Tryptophane hydroxylase
Vcl	Vitelline coat lysine
vCTB	*See* Cytotrophoblaste villeux
Verl	Vitelline envelope receptor for lysine
VMAT	Transporteur vésiculaires des monoamines
Zn	Zinc

CHAPITRE 1 :
INTRODUCTION GÉNÉRALE

1.1. Sérotonine

La sérotonine, ou 5-hydroxytryptamine (5-HT), a été caractérisée et isolée à partir du sérum bovin en 1948 (Rapport *et al.*, 1948a, Rapport *et al.*, 1948b). Rapport et ses collaborateurs étaient à la recherche d'une substance vasoconstrictrice contenue dans le sang, causant l'hypertension (Green, 2006). Son nom provient de sa présence dans le sérum (séro-) et ses propriétés tonifiantes (-tonine). Au même moment, Vittorio Erspamer décrivait les actions de l'entéramine, retrouvée dans les cellules entérochromaffines du tractus gastro-intestinal de vertébrés, dans les glandes salivaires d'octopodes et dans les branchies de mollusques (Erspamer *et al.*, 1952, Green, 2006). Son action vasoconstrictrice sur les muscles lisses et utérins a permis d'établir que l'entéramine était, en fait, la 5-HT (Erspamer *et al.*, 1952, Green, 2006, Whitaker-Azmitia, 1999).

En raison de sa similarité structurale avec l'acide D-lysergique diéthylamide (LSD) et sa découverte dans le cerveau des mammifères, un rôle de neurotransmetteur a été assigné à la 5-HT (Green, 2006, Twarog *et al.*, 1953). Elle est maintenant associée à de nombreuses fonctions comportementales, physiologiques et cognitives comme l'humeur, la mémoire, le sommeil, l'appétit et la libido (Green, 2006, Jonnakuty *et al.*, 2008). La 5-HT agit également comme hormone et agent mitogène (Azmitia, 2001, Fecteau *et al.*, 2001, Mohammad-Zadeh *et al.*, 2008, Seuwen *et al.*, 1990). Ses différentes fonctions font en sorte que la 5-HT est impliquée dans plusieurs pathologies telles que la dépression, les migraines, l'hypertension artérielle, les troubles de l'alimentation, l'Alzheimer, des troubles inflammatoires du tractus intestinal et des complications de la grossesse (Bolte *et al.*, 2001, Hoyer *et al.*, 1994, Hoyer *et al.*, 2002, Shajib *et al.*, 2015).

1.1.1. Production

La 5-HT est produite par la conversion de l'acide aminé essentiel, le L-tryptophane (L-trp), en deux étapes (Fig. 1.1). Cependant, absorbé après ingestion, le L-trp peut emprunter différentes voies, telles que la synthèse des protéines et la synthèse des kynurénines. Cette dernière voie convertie le L-trp via la tryptophane dioxygénase (TDO) ou l'indoleamine 2,3-dioxygénase (IDO)

(de Jong *et al.*, 2009) (Fig. 1.1). La biosynthèse de 5-HT est donc dépendante de la quantité de L-trp disponible.

L'ajout du groupement hydroxyle sur le cycle benzoïque du L-trp pour produire la 5-hydroxytryptophane (5-HTP) constitue l'étape cinétiquement limitante de la biosynthèse de la 5-HT (Vrana *et al.*, 1993). Cette réaction est catalysée par l'enzyme tryptophane hydroxylase (TPH), retrouvé sous deux isoformes (TPH1 et TPH2), et de cofacteurs (c.-à-d., oxygène et tetrahydropteridine) (Jonnakuty *et al.*, 2008). On observe essentiellement la TPH1 dans le système nerveux périphérique et dans les cellules entérochromaffines (Côté *et al.*, 2003). On estime qu'environ 95 % de la production de 5-HT du corps a lieu dans ces cellules du système gastro-intestinal (Furness et al., 1982, Mohammad-Zadeh et al., 2008). Cependant, la 5-HT ne peut pas franchir la barrière hématoencéphalique. Sa synthèse s'effectue donc directement dans le système nerveux central, de manière plus importante dans le noyau du raphé et dans la glande pinéale, puisque le L-trp possède la capacité de traverser cette barrière en empruntant des protéines de transport d'acides aminés. Cette voie de synthèse utilise l'isoforme TPH2 (Walther et al., 2003). Il a été suggéré que la TPH2 se retrouvait uniquement dans le système nerveux central (Gershon, 2009, Walther et al., 2003). Or, une équipe de l'Université de Melbourne a identifié la TPH2 dans le système nerveux périphérique, soit dans le plexus mésentérique (Neal et al., 2009). Notre équipe a démontré l'expression de l'ARNm et de la protéine de la TPH2 dans les cellules placentaires (Deroy *et al.*, 2011). La TPH2 a également été observée dans l'embryon de la souris (Amireault *et al.*, 2013).

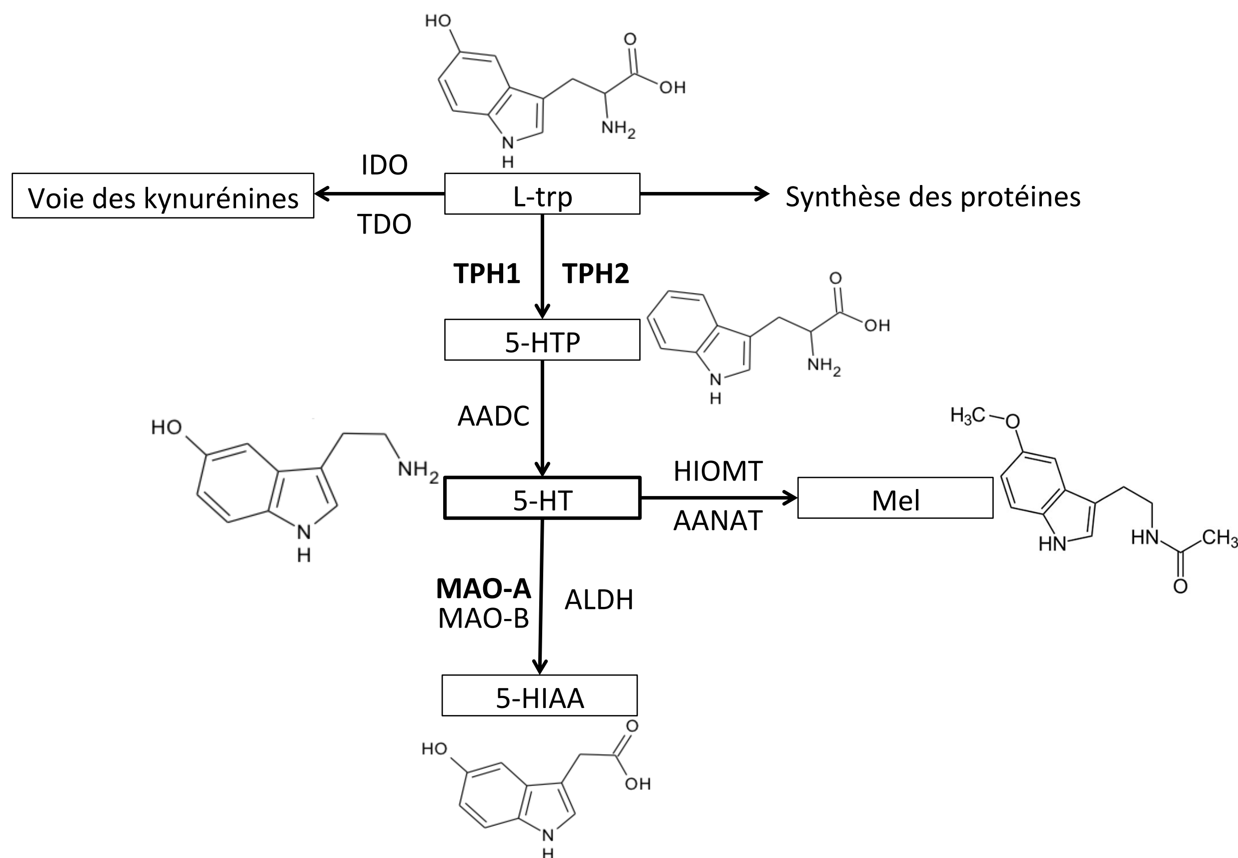

Figure 1.1 : Métabolisme de la 5-HT

L'acide aminé L-tryptophane (L-trp) issu de l'alimentation est hydroxylé par l'une ou l'autre des tryptophanes hydroxylases (TPH1 ou TPH2), résultant en le 5-hydroxytrypophane (5-HTP). Ce dernier est ensuite décarboxylé par l'acide L-amino décarboxylase (AADC) pour former la sérotonine (5-HT). La 5-HT est précurseur à la formation de la mélatonine (Mel) sous l'action successive des enzymes aralkylamine N-acetyltransferase (AANAT) et hydroxyindole O-methyltransferase (HIOMT). La 5-HT peut être dégradée sous l'action de la monoamine-oxydase (MAO) et de l'aldéhyde déshydrogénase (ALDH), résultant en la production de l'acide 5-hydroxyindol aminé (5-HIAA) qui est ensuite rejeté du corps. Le L-trp est majoritairement utilisé pour la production de protéines et est aussi impliqué dans la voie des kynurénines sous l'action des enzymes indoleamine 2,3-dioxygenase (IDO) et tryptophane dioxygénase (TDO), limitant sa disponibilité pour la biosynthèse de la 5-HT. © M Fraser

La deuxième étape de la synthèse de la 5-HT se produit sous l'action de l'acide L-amino décarboxylase (AADC) et de ses cofacteurs, les vitamines B3 et B6 et le magnésium, qui transforme la 5-HTP en retirant son groupement carboxyle, formant la 5-HT (Fig. 1.1) (Boadle-Biber, 1993, Jonnakuty *et al.*, 2008). La 5-HT, captée par les transporteurs vésiculaires des monoamines (VMAT), est ensuite emmagasinée dans les vésicules (Bottalico *et al.*, 2004). Elle peut également relarguée dans le milieu extracellulaire par ces vésicules (Fig. 1.2).

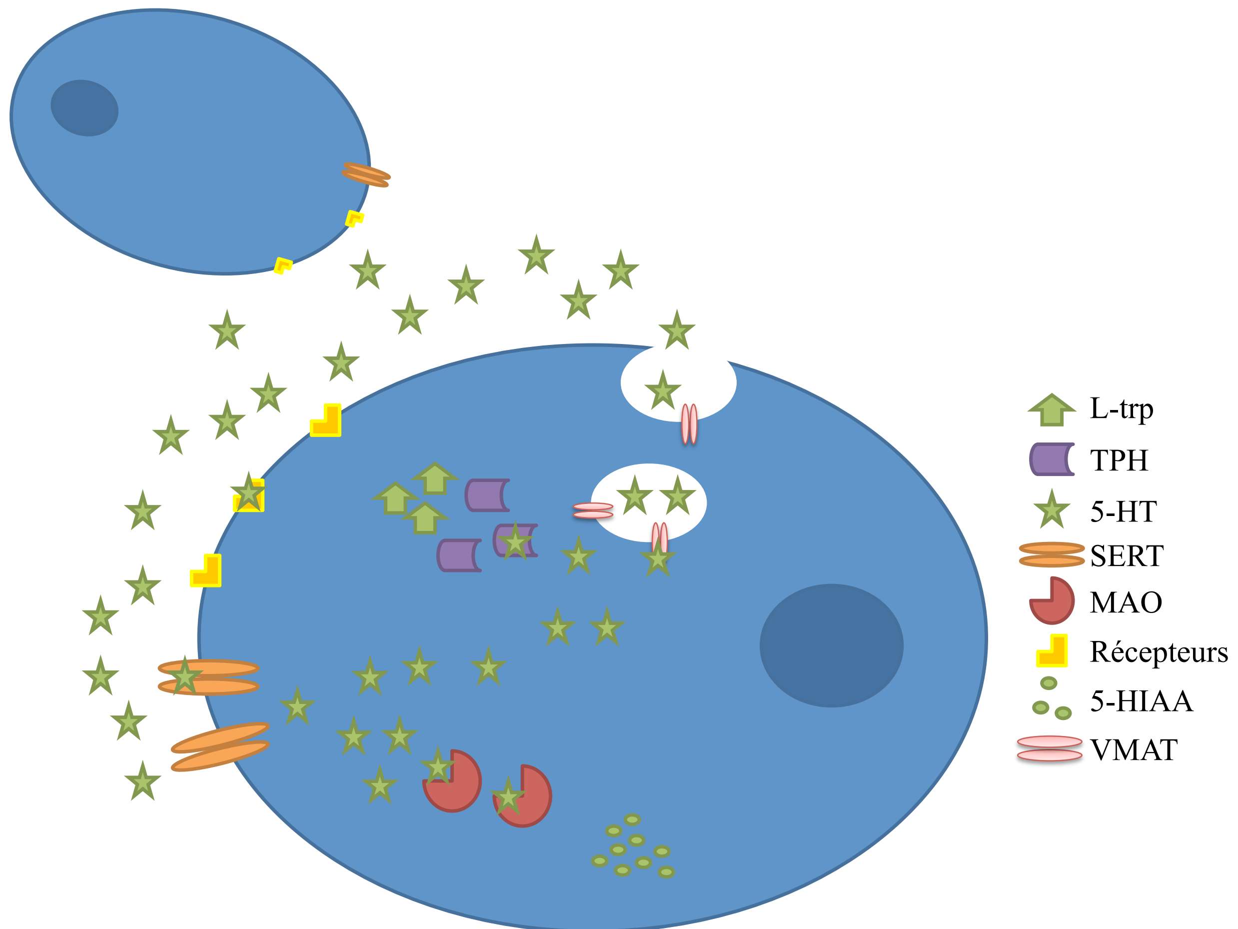

Figure 1.2 : Simplification du métabolisme principal de la 5-HT

Le L-tryptophane (L-trp) est transformé par les tryptophanes hydroxylases (TPH) pour former la sérotonine (5-HT). Cette dernière est ensuite emmagasinée dans les vésicules en empruntant les transporteurs vésiculaires des monoamines (VMAT). Ces vésicules peuvent larguer la 5-HT dans le milieu extracellulaire où la monoamine peut activer ses récepteurs de manières autocrine, paracrine et endocrine. La 5-HT extracellulaire est ensuite transportée par son transporteur spécifique (SERT). De nouveau dans le cytosol, la 5-HT peut être recyclée ou dégradée par la monoamine-oxydase (MAO), produisant un métabolite, l'acide 5-hydroxyindolacétique (5-HIAA). © M Fraser

1.1.2. Récepteurs de la sérotonine

L'action biologique de la 5-HT provient de sa liaison avec ses 17 récepteurs qui sont ubiquitaires dans le corps (Green, 2006). Ces derniers sont classés en sept familles distinctes (5-HT_1 à 5-HT_7) selon leur homologie de séquence protéique et leur structure (Fig. 1.3) (Hoyer et al., 1994, Jonnakuty et al., 2008). Unique parmi les classes, les récepteurs 5-HT_3 sont des canaux d'ions (Na^+/K) (Hoyer et al., 2002, Mohammad-Zadeh et al., 2008). Toutes les autres classes font partie de la superfamille des récepteurs à sept domaines transmembranaires qui impliquent une protéine G. À son tour, cette protéine déclenche une cascade intracellulaire qui varie selon

la classe. Il est généralement accepté que les récepteurs $5\text{-}HT_1$ et du $5\text{-}HT_5$, par le biais d'une protéine Gi/o, atténuent l'activité de l'adénosine monophosphate cyclique (AMPc) et, par conséquent, celle de la protéine kinase A (PKA) (Azmitia, 2001, Hoyer et al., 1994). Une activation de l'AMPc est toutefois observée pour les récepteurs associés à une protéine Gs, soit $5\text{-}HT_4$, $5\text{-}HT_6$ et $5\text{-}HT_7$ (Hoyer et al., 2002). Pour ce qui est des récepteurs $5\text{-}HT_2$, ils sont liés à une protéine Gq qui, par cascade signalétique, augmente l'activité de la phospholipase C (PLC) (Azmitia, 2001, Hoyer et al., 2002). De plus, ces récepteurs augmentent les niveaux intracellulaires de calcium (Ca^{2+}), ce qui augmente les activités métaboliques des cellules, ce qui mène à l'activation de la protéine kinase C et divers facteurs de transcription (Azmitia, 2001).

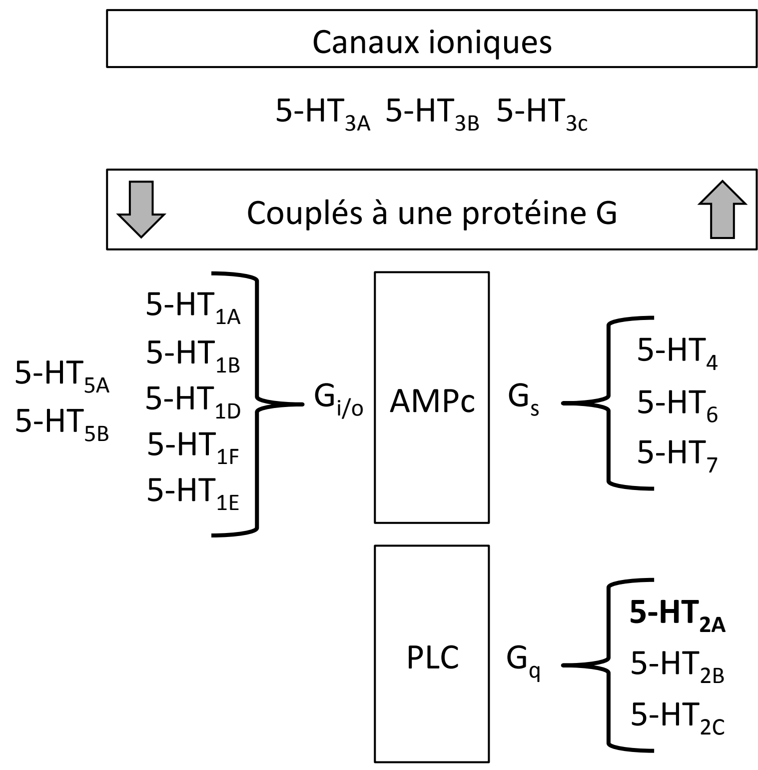

Figure 1.3 : Classification des familles de récepteurs de la sérotonine

Les récepteurs de la 5-HT sont classés en sept familles selon leur homologie de séquence protéique et leur structure. Les récepteurs $5\text{-}HT_3$ sont des canaux ioniques, tandis que les autres sont couplés à une protéine G. Adénosine monophosphate cyclique (AMPc); Phospholipase C (PLC).

1.1.3. Homéostasie de la sérotonine

En trop grande quantité, la 5-HT peut avoir des effets délétères sur l'organisme à cause de ses propriétés vasoconstrictrices (Jonnakuty *et al.*, 2008). C'est pourquoi les concentrations de 5-HT sont bien régulées par son transport, sa transformation et sa dégradation.

1.1.3.1. Transport de la sérotonine

Dès que la 5-HT est relâchée dans le plasma sanguin, elle est rapidement captée par les plaquettes sanguines où elle est stockée dans les granules denses. Celles-ci contiennent presque toute la 5-HT circulante du corps (Jonnakuty *et al.*, 2008). Le transport de la 5-HT par les cellules est principalement régulé par son transporteur spécifique (SERT ou 5-HTT) (Blakely et al., 1991). Cette protéine à 12 domaines transmembranaires nécessite le cotransport du sodium (Na^+) et du chlore (Cl^-), ainsi qu'un contre-transporteur, le potassium (K^+), selon une stœchiométrie 1 : 1 : 1 : 1 (Cool et al., 1991, Ni et al., 2006, Rudnick, 2006). Le mécanisme du transport de la 5-HT se base sur un principe symport-antiport (Fig. 1.4) (Rudnick, 2006). L'exposition du site de liaison du SERT alterne entre le milieu extracellulaire et le cytosol. Lors d'un transport symport, au moins deux substrats doivent se lier aux sites actifs afin que la protéine puisse changer de conformation. Dans le cas du SERT, le Na^+, le Cl^- et la 5-HT induisent la modification donnant accès à l'intérieur de la cellule (Fig. 1.4) (Rudnick, 2006). Lors d'un transport antiport, un seul substrat permet la remise à l'état original de la protéine. Pour le SERT, ce substrat est le K^+ (Fig. 1.4) (Rudnick, 2006). Ce transport est complexe puisqu'il doit permettre une liaison de coordination efficace de ses substrats pour laisser le changement conformationnel se produire, tout en s'assurant que ces derniers peuvent s'en dissocier facilement. Ce modèle de transport peut seulement avoir lieu si tous les substrats ont accès aux sites liants et si la conformation de la protéine n'est pas altérée (Rudnick, 2006).

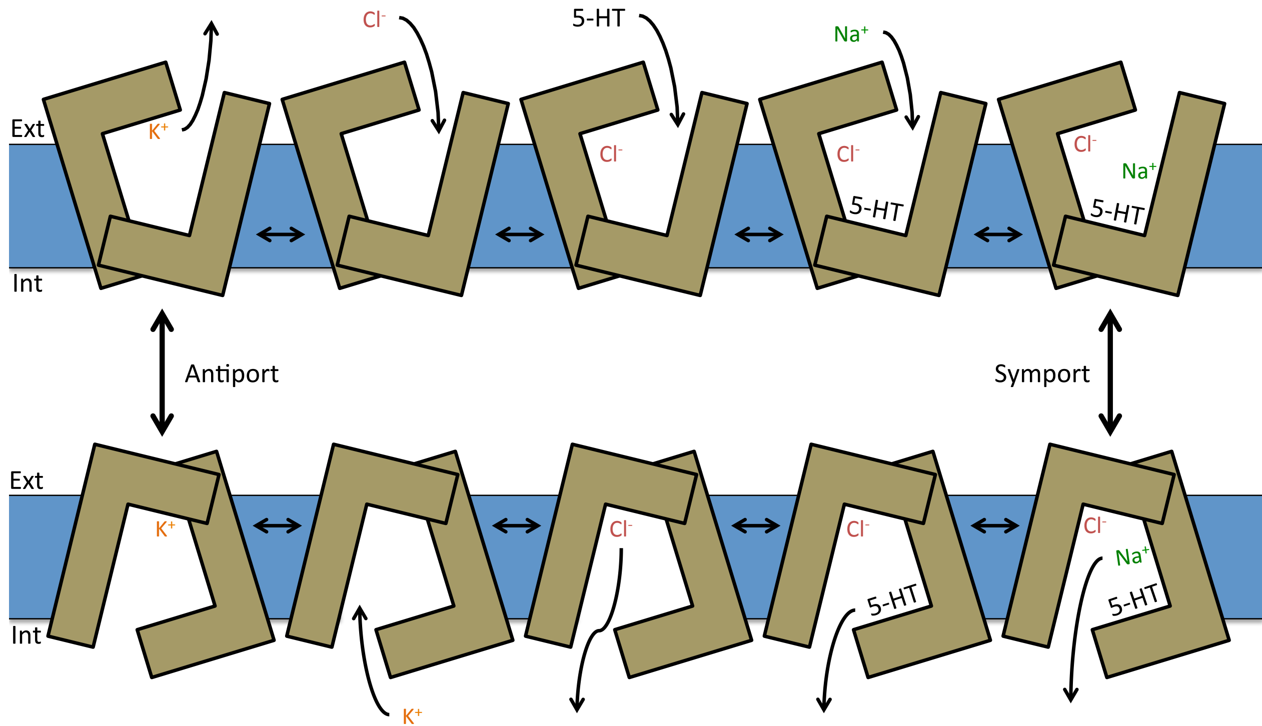

Figure 1.4 : Mécanisme suggéré du transport de la 5-HT par SERT

Le transport actif de la 5-HT vers l'intérieur de la cellule par SERT s'effectue selon un cycle symport-antiport impliquant la participation de co-transporteurs ioniques. Il est suggéré que l'ion chlorure (Cl^-), la 5-HT et l'ion sodium (Na^+) se lient respectivement au SERT. Il n'y a cependant pas d'évidence sur l'ordre de liaison des composantes. Lorsque ces liaisons sont effectuées, le changement de conformation a lieu, permettant l'ouverture de la protéine vers le cytosol. Après la dissociation des composantes, un ion potassium (K^+) se joint à la protéine pour permettre son retour à la conformation initiale. Le cycle se termine lorsque le K^+ est relâché dans le milieu extracellulaire, libérant les sites de liaisons. Le SERT est donc prêt à entreprendre un nouveau cycle.

On retrouve le SERT dans le système nerveux central, mais aussi en périphérie, tels que dans les conduits gastro-intestinaux, les artères, le cœur et le placenta (Mohammad-Zadeh *et al.*, 2008, Ni *et al.*, 2006, Viau *et al.*, 2009). Son expression est modulée par la concentration intracellulaire en Ca^{2+} (Jayanthi et al., 1994, Seimandi et al., 2013).

La 5-HT peut emprunter d'autres voies pour son transport dans les cellules. Les trois transporteurs de cations organiques (OCT1, OCT2 et OCT3) et le transporteur de monoamine de la membrane plasmique (PMTA) contribuent, dans une moindre mesure, au transport des monoamines (Bottalico *et al.*, 2004, Hansson *et al.*, 2009, Roth *et al.*, 2012). Il a été démontré dans l'hippocampe de rats que le transport de la 5-HT avait toujours lieu malgré l'utilisation d'inhibiteurs sélectifs de la recapture de la 5-HT (ISRS) (Courousse et al., 2015, Daws et al., 2005). Ceci suggère que ces autres transporteurs peuvent aussi transporter la sérotonine

(Courousse et al., 2015). Ces transporteurs sont présents dans divers tissus, dont l'intestin, le cerveau, l'utérus, le placenta et le cœur (Hansson et al., 2009).

1.1.3.2. Transformation et dégradation

La 5-HT peut être soit recyclée en se faisant capter à nouveau par les VMAT (Fig. 1.2), soit elle emprunte une voie de transformation ou de dégradation (Bottalico et al., 2004). La 5-HT est le précurseur de la mélatonine (Mel). Sous actions successives des enzymes aralkylamine N-acétyltransférase (AANAT) et hydroxyindole O-méthyltransférase (HIOMT) (Fig. 1.1), la 5-HT est transformée en Mel. La production de la Mel était autrefois assignée uniquement à la glande pinéale (Devika Warrier *et al.*, 2015, Tyce, 1990). Cependant, il est maintenant établi que le système Mel se retrouve également dans d'autres régions du cerveau, le tractus gastro-intestinal, les organes reproducteurs, la peau, les plaquettes et le placenta (pour revue Sagrillo-Fagundes *et al.* (2016)).

La voie majoritaire de dégradation de la 5-HT se fait par la monoamine-oxydase (MAO) (Fig. 1.1 et 1.2). Avec l'aide de l'aldéhyde déshydrogénase (ALDH), il en résulte le métabolite acide 5-hydroxyindol acétique (5-HIAA) qui est ensuite éliminé par l'urine (Fig. 1.2) (Jonnakuty *et al.*, 2008). La MAO possède deux isoformes (MAO-A et -B) exprimées dans les mitochondries de certains types cellulaires (Shih *et al.*, 1999). L'expression et l'activité des isoformes de la MAO varient de manière préférentielle selon l'organe dans lequel ont les retrouves (Billett, 2004). Ainsi, on constate que les cellules du cerveau et les plaquettes contiennent plus de MAO-B, contrairement au placenta qui possède presque uniquement la MAO-A (Andrade *et al.*, 2015, Billett, 2004). De plus, malgré leur structure similaire, les isoformes de la MAO ont des affinités spécifiques plus grandes envers certains substrats (Youdim et al., 2004). Par exemple, la 5-HT est dégradée dix fois plus rapidement par la MAO-A que par la MAO-B (Youdim *et al.*, 2006, Youdim *et al.*, 2004).

1.1.4. Grossesse et sérotonine

L'ensemble du système 5-HT est présent dans le placenta, organe essentiel au bon déroulement de la grossesse. Ceci suggère une implication importante de la 5-HT lors du développement placentaire et fœtal (Arseneault et al., 2005, Balkovetz et al., 1989, Bonnin et al., 2011a, Bottalico et al., 2004, Deroy et al., 2011, Huang et al., 1998, Mitchell et al., 1983, Oufkir et al., 2010, Sonier et al., 2005, Vaillancourt et al., 1994, Viau et al., 2009). En outre, la 5-HT stimule la prolifération des cellules placentaires tant chez les cellules bovines que chez les

cellules de choriocarcinome humain (Fecteau et al., 2001, Oufkir et al., 2010, Sonier et al., 2006). Notre équipe de recherche a démontré par l'action de l'hydrochlorure de 2,5-diméthoxy-4-indoamphytamine (DOI), un agoniste sélectif du récepteur 5-HT_{2A}, une augmentation de la viabilité, de la prolifération et de la migration des cellules BeWo issues d'un choriocarcinome placentaire (Arseneault et al., 2005, Oufkir et al., 2010, Sonier et al., 2005). Ce récepteur et les récepteurs 5-HT_{1A} et 5-$HT_{2(B\ et\ C)}$ sont exprimés dans le placenta (Arseneault et al., 2005, Huang et al., 1998, Sonier et al., 2005, Vaillancourt et al., 1994, Viau et al., 2009).

La 5-HT est essentielle à l'embryogenèse (Buznikov et al., 2001) ainsi qu'au développement des neurones (Bonnin *et al.*, 2011a, Côté *et al.*, 2007, Seuwen *et al.*, 1990) et des cellules cardiaques fœtales (Côté *et al.*, 2003, Sari *et al.*, 2003). Son rôle dans le développement précoce de l'embryon est supporté par l'expression de récepteurs et du transporteur avant même l'innervation (Bonnin et al., 2011a, Buznikov et al., 2001). Il a été suggéré que la 5-HT nécessaire au développement du cerveau est de source exogène puisque l'enzyme de synthèse de la 5-HT (TPH2) n'est pas exprimée avant le 10e jour embryonnaire chez la souris (Bonnin *et al.*, 2011a, Côté *et al.*, 2007). De plus que la TPH1, enzyme retrouvée dans les cellules gastriques, n'est pas exprimée avant le jour 15 (Bonnin et al., 2011a, Booij et al., 2015).

Les équipes de Côté et Levitt suggèrent que la 5-HT embryonnaire est d'origine maternelle (Bonnin *et al.*, 2011a, Côté *et al.*, 2007). Ceci est supporté par l'expression du SERT dans diverses cellules placentaires, dont le syncytiotrophoblaste, le cytotrophoblaste villeux et les capillaires fœtaux (Balkovetz et al., 1989, Bottalico et al., 2004, Prasad et al., 1996, Viau et al., 2009). Cependant, les fœtus de mères qui n'ont pratiquement pas de 5-HT sanguine, suite à l'inactivation du gène *SERT/SCL6A4*, ont des concentrations de 5-HT comparables à celles de fœtus de mère de type sauvage (Bonnin et al., 2011a). Cette étude jette un doute sur la provenance maternelle de la 5-HT, mais n'exclut pas la participation de la 5-HT maternelle au développement de l'embryon (Bonnin et al., 2011b). De plus, une expression et une grande activité de la MAO-A au niveau du placenta suggèrent une dégradation de la 5-HT maternelle (Auda et al., 1998, Billett, 2004). Le SERT jouerait alors un rôle dans la régulation de l'apport sanguin vers le placenta en assurant la clairance de la 5-HT qui possède une action vasoconstrictrice sur les vaisseaux (Bottalico et al., 2004). Cependant, l'expression des enzymes TPH1 et TPH2 et une production de la 5-HT dans les cellules placentaires humaines ont été démontrées entre autres par notre équipe (Deroy et al., 2013, Deroy et al., 2011, Huang et al., 1998). Chez les souris, la production de novo de 5-HT par la TPH1 et un transfert de la monoamine vers le fœtus ont été démontrés (Bonnin et al., 2011a). Ceci suggère donc que la

5-HT nécessaire au développement fœtal provient, entre autres, du placenta. Par conséquent, un dérèglement du système 5-HT placentaire peut être associé à divers troubles de la grossesse et à une altération du développement fœtal. On observe, entre autres, une augmentation des concentrations de 5-HT lors d'une prééclampsie (Bolte et al., 2001). Un défaut d'invasion associé à une hypertension et une protéinurie caractérise cette pathologie de la grossesse (Tsatsaris et al., 2008). Notre équipe a observé une diminution de l'expression protéique et génique du récepteur *HTR2A*/5-HT_{2A} et du *SCL6A4*/SERT dans les placentas issus de grossesses de diabète gestationnel comparativement à des grossesses sans pathologie (Viau et al., 2009).

Les souris femelles dont le gène *TPH1* a été inactivé donnent naissance à des souriceaux de plus petite taille que ceux de femelles de type sauvage (Côté *et al.*, 2007). Une perturbation des niveaux de 5-HT entraîne également des malformations cardiaques et neuronales (Bonnin *et al.*, 2011a, Côté *et al.*, 2007, Côté *et al.*, 2003). Les effets sur le développement du cerveau sont associés à des problèmes neurologiques après la naissance, tels que l'anxiété (Beaulieu et al., 2008, Hendricks et al., 2003) et des troubles d'attentions avec ou sans hyperactivité (Halmoy et al., 2010). De plus, une utilisation d'inhibiteurs sélectifs de la recapture de la 5-HT (ISRS), des antidépresseurs, lors de la grossesse est associée avec des troubles du spectre autistique (Boukhris et al., 2015, Favreliere et al., 2010, Hviid et al., 2014, Sato, 2013). De tels effets d'une dérégulation de l'homéostasie lors de développement fœtal, qui se reflètent après la naissance et qui perdurent tout au long de la vie, sont associés au phénomène de la programmation fœtale décrite par Barker et al. (1993) (Bonnin et al., 2011b, Paquette et al., 2013, St-Pierre et al., 2015).

1.1.5. Mollusques et sérotonine

La 5-HT est présente dans divers animaux, dont des amphibiens, les octopodes, les rats et les mollusques (Erspamer et al., 1952, Green, 2006, Rapport et al., 1948a, Rapport et al., 1948b, Twarog et al., 1953). La signalisation de la 5-HT est grandement conservée entre les espèces où elle agit de manière similaire que chez l'humain (Borue et al., 2007, Pani et al., 1998). En outre, on retrouve la 5-HT dans les branchies, le système nerveux, le muscle adducteur et les glandes digestives des mollusques bivalves (Almeida et al., 2003, Dietz et al., 1985, Stefano et al., 1975, Sweeney, 1968, Twarog, 1954). En comparant les sections partielles du *Scl6a4*/*SERT* cloné de mollusques, tels que l'ellipso pointue (*Elliptio dilatata*) et l'escargot des buissons (*Cepaea nemorlais*), avec d'autres organismes, on constate une grande correspondance

(Caveney et al., 2006). Les récepteurs de la 5-HT observés chez les mollusques sont semblables à certains retrouvés chez les humains (Tanabe et al., 2010, Tierney, 2001). Grâce à une étude d'effets d'agonistes et d'antagonistes, il apparaît que les moules possèdent des récepteurs analogues (récepteurs-like) aux récepteurs des classes 5-HT1 et 5-HT2 (Fong *et al.*, 1993b, Rand-Weaver *et al.*, 2013).

La 5-HT joue un rôle primordial dans la régulation du métabolisme de la moule. Elle est, entre autres, impliquée dans le battement des cils des branchies (Aiello, 1965, Aiello, 1962, Beiras *et al.*, 1995b, Carroll *et al.*, 2007), la filtration par le mouvement du siphon (Ram *et al.*, 1999, Salanki *et al.*, 1990) et la relaxation des fibres musculaires adducteurs (Almeida *et al.*, 2003, Gies, 1986), ainsi que du byssus (Muneoka *et al.*, 1991). La 5-HT est également associée à la reproduction des mollusques, principalement à la différenciation sexuelle, la gamétogénèse et la ponte (Fong *et al.*, 2003, Gagné *et al.*, 2003, Gibbons *et al.*, 1984, Wang *et al.*, 2014). Ainsi, une altération du système 5-HT de la moule peut entraîner des problèmes de reproduction. Par exemple, une exposition à de faibles concentrations de fluvoxamine, de paroxétine et de fluoxétine, des ISRS, ainsi qu'au métabolite de la fluoxétine, la norfluoxétine, induit la ponte chez la moule zébrée (*Dreissena polymorpha*) (Fong *et al.*, 2008, Fong, 1998). L'utilisation d'antagonistes de récepteurs humains 5-HT_2 bloque l'induction de la ponte par les ISRS, supposant une implication d'un récepteur 5-HT_2-like dans la reproduction de la moule zébrée (Fong *et al.*, 2003).

1.2. Métaux

Naturellement enfouis dans les sols, les océans et les roches, les métaux ne causent généralement pas de risque pour la santé. Libérés dans l'environnement, ils peuvent causer une contamination toxique pouvant entraîner d'importantes conséquences sur l'ensemble de l'écosystème (Burger *et al.*, 2007, Fraser, 2010). Les phénomènes naturels, tels que les feux de forêt, les éruptions volcaniques et les inondations, et l'activité anthropique contribuent à cette contamination environnementale (Garrett, 2000, Mandal *et al.*, 2006). L'exploitation de mines, de fonderies et l'utilisation de combustibles fossiles à des fins énergétiques ne sont que quelques exemples d'industries émettrices de métaux (Hilderbrand, 1984, Miller *et al.*, 2000, Newhook *et al.*, 2003).

1.2.1. Métaux et exposition humaine

Certains métaux sont essentiels au maintien de l'homéostasie des systèmes biologiques des organismes. Ces oligo-éléments, tels que le Ca, le zinc (Zn) et le manganèse (Mn), participent aux actions des enzymes et peuvent participer à l'homéostasie des cellules et aux actions des enzymes (pour revue ATSDR (2004b)). Ils sont, entre autres, indispensables au bon fonctionnement des systèmes immunitaire, nerveux, reproductif et endocrinien (Favier *et al.*, 2005, Walsh *et al.*, 1994, WHO, 1983, WHO, 1998, WHO, 2001). Nonobstant leurs effets essentiels, les oligo-éléments peuvent engendrer des effets semblables à ceux des métaux toxiques, tels que le plomb (Pb) et le cadmium (Cd), lorsque leurs concentrations dans l'organisme sont trop élevées (WHO, 1983, WHO, 1998, WHO, 2001). Les effets varient selon la durée et la dose de l'exposition. Une exposition à de fortes doses toxique, c'est-à-dire une exposition aiguë, entraîne des effets néfastes immédiats sur la santé pouvant mener à la mort. Dans ces cas extrêmes, rarement observés et plutôt ponctuels, il est souvent possible de faire le lien direct entre la substance nocive et les effets observés sur la santé, tels des étourdissements, des maux de tête et des pertes de conscience (Nordberg et al., 2015). Il est cependant beaucoup plus difficile de faire l'association entre les maladies et les substances toxiques lors d'une exposition qui s'échelonne sur une longue période de temps à des concentrations faibles, des expositions chroniques ou qui ne diffère pas de la population générale (Nordberg et al., 2015). Par exemple, une exposition continue à de faibles niveaux de Mn et au Pb est associée à des altérations du développement cognitif et comportemental (Bouchard *et al.*, 2011, Chandra *et al.*, 1981, Finley, 2004, Grandjean *et al.*, 2006, Neal *et al.*, 2013, Verity, 1999, WHO, 1977). Une exposition chronique au Cd peut mener à des problèmes rénaux et à certains types de cancers, dont celui de la prostate et des reins (Jarup, 2003, Nawrot *et al.*, 2006, Vinceti *et al.*, 2007).

1.2.2. Métaux et grossesse

Les métaux accumulés et stockés dans le corps peuvent être mobilisés sous l'action de changements physiologiques importants, notamment durant la grossesse, où le métabolisme est augmenté à cause de la demande accrue en nutriment. Par exemple, le fœtus ayant besoin d'une grande quantité de Ca pour assurer son bon développement, la demande en Ca de la mère est augmentée, ce qui peut avoir comme effet de libérer le Ca des os, rendant le Pb et le Cd, stocké dans les os, à nouveau biodisponibles (Anway *et al.*, 2005, Dorea *et al.*, 2006,

Gulson *et al.*, 1997, Lafond *et al.*, 2004, Thomas *et al.*, 2015). Une exposition aux métaux durant la grossesse est associée, par des stress oxydatifs, à un risque accru de pathologies, comme la prééclampsie et le diabète gestationnel (Laine *et al.*, 2015, Motawei *et al.*, 2013, Shapiro *et al.*, 2015, Vigeh *et al.*, 2006). Les métaux peuvent également interférer avec le fonctionnement du placenta, traverser la barrière placentaire et ainsi, directement et indirectement, altérer le développement du fœtus (Genuis, 2006, Grandjean *et al.*, 1997, Lafond *et al.*, 2004, Malassiné *et al.*, 2000). En outre, des diminutions du poids à la naissance, de la période de gestation et de la circonférence de la tête du nouveau-né ont été associées à une exposition durant la grossesse à de faibles concentrations de Pb et au Cd (Esteban-Vasallo *et al.*, 2012, Falcon *et al.*, 2002, Goyer, 1990, Osman *et al.*, 2000, Ostrea *et al.*, 2002). Des effets d'une exposition *in utero* aux métaux peuvent aussi se manifester après la naissance (appelé programmation fœtale), tels que des altérations au niveau du développement cognitif, neurocomportemental et psychomoteur (Dorea *et al.*, 2006, Goyer, 1990, Grandjean *et al.*, 2006, Hu *et al.*, 2006, Ostrea *et al.*, 2002, Rice *et al.*, 2000, Takser *et al.*, 2003, Walker, 2000, Wasserman *et al.*, 2000, Yu, 2005).

1.2.3. Métaux et mollusques

Bien que les mollusques soient très utilisés pour suivre la contamination environnementale en raison de leur propension à bioaccumuler des métaux, peu d'études s'intéressent aux effets sur ses systèmes biologiques de cette contamination (Zuykov *et al.*, 2013). Néanmoins, les métaux peuvent interférer avec le métabolisme des bivalves (Brahim Errahmani *et al.*, 2014). Ainsi, les métaux altèrent la respiration, l'activité des cils des branchies, la membrane lysosomale, la reproduction et la gamétogénèse, et perturbent le fonctionnement et l'expression de certains enzymes (Beiras *et al.*, 1995a, Brown *et al.*, 1972, Caricato *et al.*, 2010, Viarengo *et al.*, 2000). Le mercure cause une diminution de la croissance et une inhibition de la capacité à nager de l'embryon de *Mytilus galloprovincialis* (Beiras *et al.*, 1995a). Une exposition à de fortes concentrations de cuivre diminue la consommation en oxygène de *Mytilus edulis* (Brown *et al.*, 1972). Chez *Mytilus galloprovincialis*, de fortes concentrations de Cd augmentent l'activité de l'anhydrase carbonique, l'enzyme qui assure la transformation du gaz carbonique en acide carbonique (Caricato *et al.*, 2010). Le système de défense immunitaire des moules, principalement basé sur la phagocytose, est aussi diminué sous l'action de métaux (Brousseau et al., 2000, Fraser et al., 2014, Gagnaire et al., 2004, Rault et al., 2013, Sauvé et al., 2002) (voir Annexe A3).

1.2.4. Métaux et sérotonine

Plusieurs études démontrent une perturbation du système 5-HT par le Mn, le Pb et le Cd (Tableau 1.1). Celles-ci se concentrent majoritairement sur les effets des métaux dans le système nerveux central de modèles animaux. Chez le rat, il a été notamment observé une diminution des concentrations de 5-HT cérébrale à la suite de l'exposition aux trois métaux (Bonilla et al., 1984, Lafuente et al., 2003, Nation et al., 1989, Widmer et al., 1991). Cette diminution peut provenir d'une réduction de l'activité des TPH (Khan et al., 2000, Ogawa et al., 2006). Cependant, deux études chez les rats exposés au Pb ou au Mn démontrent une augmentation du taux de 5-HT dans le cerveau (Chand Basha et al., 2014, Subhash et al., 1991). Au vu des résultats, il semble que l'action des métaux sur le système 5-HT est complexe. Par exemple, l'activité de la MAO-A des planaires (*Dugesia japonica*) est augmentée dans leur queue à la suite d'une exposition au Cd, alors que l'activité de la MAO-B y est diminuée (Wu et al., 2015). Une seule étude porte sur les effets des métaux sur les récepteurs de la 5-HT. En effet, Harikumar *et al.* (2001) montrent une inhibition de la liaison de la 5-HT avec le récepteur 5-HT_{1A} dans l'hypothalamus des bovins.

Aucune étude n'a été répertoriée chez la moule bleue (*Mytilus edulis*) (Tableau 1.1). Cependant, chez les moules brunes (*Perna perna*), exposées pendant 24 h au Pb ou au Cd, une diminution des taux de 5-HT dans les muscles et la glande digestive a été observée (Almeida et al., 2003). Ceci est en accord avec les résultats obtenus par Salanki et Hiripi qui ont démontré une diminution du taux de 5-HT dans les ganglions de la moule anodonte (*Anodonta cygnea*) après une exposition au Pb et au Cd (Salanki et al., 1990). De plus, une exposition à une concentration de 100 µM de Pb ou de Cd diminue le transport de la 5-HT (Salanki et al., 1990).

Bien que les résultats obtenus chez des modèles animaux aient mis en évidence que les métaux altèrent le système sérotoninergique, très peu d'études montrent leurs effets sur le système sérotoninergique humain (Tableau 1.1). L'étude de Takser *et al.* (2003) sur une cohorte de femmes enceintes et leurs bébés, démontre une corrélation négative entre les taux de Mn du sang maternel et les niveaux de 5-HIAA dans le sang de cordon. Cette association peut être expliquée par une diminution du taux de 5-HT et/ou de son transfert par le placenta ou par une inhibition de la MAO (Takser et al., 2003). Par contre, il a récemment été déterminé que les taux de Mn retrouvés dans le sang maternel et celui du cordon sont corrélés positivement à l'activité des MAO placentaires (Abdelouahab et al., 2010). Des corrélations négatives entre les de Pb et de Cd du sang de cordon et l'activité des MAO placentaire sont y aussi observées (Abdelouahab et al., 2010).

Tableau 1.1 : Effets du Cd, Pb et Mn sur le système 5-HT

Métal	Organisme	Composante	Organes/Structures	Concentration	Temps d'exposition	Résumé des effets	Références
Cd	Planaires *(Dugesia japonica)*	• 5-HT • Activité MAO-A/B	Organisme divisé en 2 (tête & queue)	0,63 mg/L (~5600 nM) dans l'eau	1, 4, 7 jours	• ↓ 5-HT dans la tête à 1 jour • ↑ MAO-A à 7 jours (queue) • ↓ MAO-B à 1 jour (queue & tête)	Wu *et al.* (2015)
Cd	Rats mâles	• 5-HT • Ratio 5-HIAA/5-HT	Hypothalamus	25 ppm (~220 µM) dans l'eau de consommation	Toutes les 4 h pendant 24 h	• ↓ 5-HT dès les 4 premières heures • ↓ Ratio après 12 h	Lafuente *et al.* (2003)
Cd	Rats mâles (adolescent & adulte)	Ratio 5-HIAA/5-HT	Hypothalamus	50 ng/mL (~450 nM) dans l'eau de consommation	30 jours	• Ratio varie selon la région de l'hypothalamus et de l'âge des rats : – Ado : ↑ dans 2 régions, ↓ dans 1 et ne change pas dans 1. – Adulte : ↑ dans 2 régions et ne change pas dans 2	Lafuente *et al.* (2001)
• Cd • Pb • Mixte	Rats mâles	• 5-HT • 5-HIAA • Ratio 5-HIAA/5-HT	Cerveau	• 100 ppm Cd (~990 µM) • 500 ppm Pb (~2,4 mM) dans la nourriture	60 jours	• ↓ 5-HT néostriatum & noyau accumbens • ↓ 5-HIAA néostriatum & cortex frontal • ↓ Ratio cortex frontal • Effets variables de l'exposition mixte	Nation *et al.* (1989)
• Cd • Pb	Moules brunes *(Perna perna)*	5-HT	• Muscle • Glande digestive	• 200 µg/L Cd (~1800 nM) • 200 mg/L Pb (~960 mM)	12, 24, 72 et 120 h	• Muscle : ↓ à 24 et 72 h. Rétablissement à 120 h. • Glande digestive : ↓ à 72h. Rétablissement pour Cd à 120 h	Almeida *et al.* (2003)
• Cd • Pb	Anodonte des cygnes *(Anadonta cygnea)*	• 5-HT • Recapture	Ganglions	• 1000 nM (atteint sur 8 h) • 10 et 100 µM (recapture)	• Dès l'atteinte de la concentration et après 8, 14, 20, 32 et 40 h • Recapture : 10 min	• ↓ 5-HT à 14, 20 h (2 métaux) et 20, 32 h pour Pb • ↓ Recapture à 100 µM	Salanki *et al.* (1990)
Pb	Tambours brésiliens *(Micropogonias undulatus)*	• Activité TPH • Activité MAO	Hypothalamus	15 mg/kg poids par jours (~72 nmol/kg poids par jours)	30 jours	• ↓ Activité TPH • ↑ Activité MAO	Khan *et al.* (2000)
Pb	Poisson-zèbre *(Danio rerio)*	• Activité MAO • ARNm (RT-PCR)	Cerveau	50 µg/L (241 nM) dans l'eau	24 et 72 h	• ↓ Activité à 24 h, mais pas à 72 h • Pas de changement pour ARNm	Senatori *et al.* (2009)
Pb	Guppy (*Poecilia reticulata)*	• Activité MAO • ARNm	Cerveau	50 µg/L (241 nM) dans l'eau	24 et 72 h	• ↑ Activité à 24 h, mais pas à 72 h • Pas de changement pour ARNm	Senatori *et al.* (2009)

↓ Diminution ; ↑ Augmentation

Tableau 1.1 : Suite

Métal	Organisme	Composante	Organes	Concentration	Temps d'exposition	Résumé des effets	Références
Pb Lait maternel	Rats mâles	Activité MAO	Cerveau	0,2 et 1% (m/v) (~10 et 50 mM) dans l'eau de la mère	Naissance jusqu'au jour 21	↓·Activité à 1%, mais pas significative à 0,2%	Devi *et al.* (2005)
Pb Lait maternel	Rats	• 5-HT • Activité MAO	Cerveau	0,2 (m/v) (~10 et 50 mM) Eau de la mère	Naissance jusqu'au 45 jours et 4, 12 et 18 mois	• ↑·5-HT, sauf cortex (4 et 12 mois) et cervelet (12 mois) • ↓·Activité MAO, sauf cortex (4 et 12 mois)	Chand Basha *et al.* (2014)
Pb Exposition pré- et post-natale	Rats 28 jours	• 5-HT • 5-HIAA	Cerveau	2,5 g/L (~12 mM) Valeur réelle 1363 ppm (~6,6 mM)	Début de la grossesse jusqu'aux jours 6 et 28 après la naissance	• ↓·5-HIAA au jour 6 mâles (néostriatum) et femelle (tronc cérébral) • ↓·5-HT au jour 28 dans le tronc cérébral (2 sexes)	Widmer *et al.* (1991)
Mn	Bovins	Liaison 5-HT$_{1A}$R	Protéine de l'hippocampe	De 1 à 1000 mM	1 heure	↓·Liaison du ligand avec le récepteur à 1 mM	Harikumar *et al.* (2001)
Mn	Rats males	• 5-HT • MAO	Cerveau	0,54 mg/mL (~10 mM)	90 jours	• ↑·5-HT dans cortex • ↓·MAO dans cervelet et cortex • ↑·MAO dans hippocampe et medulla	Subhash *et al.* (1991)
Mn	Rats males	• 5-HT • 5-HIAA	Cerveau (striatum)	10 et 15 mg MnCl$_2$/Kg (~80 et 120 nmol/Kg)	15 jours	• Pas de changement 5-HT • ↓·5-HIAA à 15 mg/Kg	Seth *et al.* (1981)
Mn	Rats	• 5-HT • 5-HIAA	Cerveau	0,1 mg/mL (~ 2 mM) eau consommation	8 mois	• ↓·5-HT dans mésencéphale • ↓·5-HIAA dans hippocampe	Bonilla *et al.* (1984)
Mn	Souris mâles	• 5-HT • 5-HIAA	Cerveau (Striatium)	0,4 g/L (~ 7 mM) eau consommation	8 semaines	• Pas de changement pour 5-HT • ↑·5-HIAA	Krishna *et al.* (2014)
Mn	Humain	Activité TPH	Clone à partir d'un cerveau fœtal	0,1 – 1000 µM	10 min	• Inhibition TPH1 à partir de 0,5 µM • Inhibition THP2 à partir de 10 µM	Ogawa *et al.* (2006)
Mn Sang maternel	Humains	5-HIAA	Sang fœtal	Moyenne 20,4 µg/L (~370 nM)	cohorte	Corrélation négative (↓)	Takser *et al.* (2003)
Mn Pb Cd sang maternel/ cordon	Humains	Activité MAO	Placenta	Moyenne (µg/L) • Mère-10,5 (~190 nM) • Cordon-31,2 (~570 nM)	cohorte	• Mn : Corrélation ↑ mat. & cordon • Pb : Corrélation ↓ cordon • Cd : Corrélation ↓ cordon	Abdelouahab *et al.* (2010)

↓·Diminution ;·↑·Augmentation

1.2.2. Mécanisme d’action

L’un des mécanismes d’action majoritaire des métaux sur les systèmes biologiques est leur capacité d’interagir avec les métaux essentiels (ATSDR, 2004a, ATSDR, 2004b). Le Pb et le Cd, par exemple, peuvent mimer le Ca et perturber son transport dans le placenta (Lafond et al., 2004, Tchounwou et al., 2012). Les protéines à doigt de Zn sont aussi affectées par le Pb et le Cd ce qui peut perturber les facteurs de transcription de l’acide désoxyribonucléique (ADN) (ATSDR, 2004a, Lin et al., 1997, Zawia et al., 2000).

Les métaux ont une grande affinité avec certains groupes fonctionnels des protéines, tels que les amines et les thiols (Disbudak et al., 2002). Ils peuvent ainsi se lier aux enzymes, modifier leurs configurations et entrer en compétition avec les substrats lorsqu’ils sont liés aux sites actifs (ATSDR, 2004b, Disbudak et al., 2002, Zawia et al., 2000). D’un autre côté, cette affinité permet à certaines protéines de séquestrer les métaux limitant leurs effets toxiques. La métallothionéine (MT), riche en groupements sulfhydryles, permet de complexer de nombreux métaux essentiels ou toxiques, tels que le Mn, le Zn, le Hg, le Cd et le Pb (ATSDR, 2004b, Sterner, 1999, Viarengo et al., 1997, Yu, 2005). De ce fait, elle permet de limiter la production d’espèces réactives de l’oxygène (ROS) induites par les métaux toxiques (Tchounwou et al., 2012).

La spéciation des métaux joue un rôle important sur leur biodisponibilité, et ainsi sur leur potentiel toxique. Le Mn est généralement retrouvé sous forme d’oxyde dans la nature, ce qui limite sa solubilité. Seulement 5 % du Mn ingéré est absorbé par l’organisme (Mergler, 1996). Dans le corps humain, le Mn est généralement sous forme inorganique (Michalke et al., 2014, Pearson et al., 2005). Cependant, sous sa forme organique comme le méthylcyclopentadiényle tricarbonyle de Mn (additif à l’essence) sa durée de vie dans l’organisme est augmentée (Michalke et al., 2007, Zheng et al., 2000).

On retrouve surtout le Pb sous une forme inorganique qui a une durée de vie de plus de vingt ans dans le corps humain. Cependant, sous forme organique, comme le tetraéthylplomb, le Pb est dix fois plus toxique que sous sa forme inorganique. Les formes organiques peuvent être métabolisées par le corps (Baxter et al., 1995).

Le Cd possède un seul état d’oxydation ce qui ne lui permet pas de créer de molécules organiques. Le principal moyen d’exposition s’effectue par la cigarette. Une fois absorbé, il est rapidement lié à la MT où il est séquestré principalement dans les reins (Astruc, 1986, Bernard, 2016, Nakamura *et al.*, 2012).

1.3. Modèles d’étude

Le placenta humain et les moules bleues offrent un potentiel indéniable pour l’étude des effets de la contamination environnementale sur le système 5-HT. En plus de tous deux posséder le système 5-HT, impliquée dans la reproduction (voir section 1.1.4 et 1.1.5), ils sont facilement accessibles et peuvent être obtenus de façon non invasive. De plus, ils ont l’aptitude de refléter les concentrations de contaminants retrouvés dans leur environnement respectif (Fraser *et al.*, 2011, Iyengar *et al.*, 2001a, Iyengar *et al.*, 2001b, Myren *et al.*, 2007).

En effet, les moules sont largement utilisées comme espèces sentinelles afin de déterminer la contamination locale en métaux (Chase *et al.*, 2001, Fraser *et al.*, 2011, Walker *et al.*, 2013). Elles possèdent toutes les qualités nécessaires aux espèces sentinelles : elles sont tolérantes à la contamination, sédentaires et abondantes (Fraser, 2010). Afin de capter leur nourriture, les moules filtrent d’importantes quantités d’eau, ce qui les expose également aux contaminants présents dans l’eau. Ainsi les moules sont des outils intéressants pour déterminer les taux de contamination de la colonne d’eau (Chase et al., 2001, Fraser, 2010, Phillips, 1976). Malgré tout, encore peu d’études s’intéressent au potentiel des moules comme indicateur d’effets des contaminants sur leur santé (Zuykov *et al.*, 2013).

Le placenta est exposé à la contamination environnementale par le sang maternel. De plus, le Mn, le Cd et le Pb peuvent s’accumuler dans le placenta (Kippler *et al.*, 2010, Piasek *et al.*, 2016, Reichrtovâ *et al.*, 1998, Takser *et al.*, 2003). Au vu de ces caractéristiques, il semble que le placenta soit un outil permettant de déterminer le taux de contamination (maternel et fœtal) et les potentiels effets de celle-ci. Bien qu’il soit facile de l’obtenir sans intervention invasive, puisqu’il est habituellement jeté après l’accouchement, le placenta est encore très peu utilisé (Iyengar *et al.*, 2001a, Iyengar *et al.*, 2001b, Piasek *et al.*, 2016). À partir des tissus placentaires obtenus après l’accouchement, les cellules trophoblastiques sont isolées et mises en culture primaire afin d’étudier les effets des contaminants (Lanoix *et al.*, 2008, Sagrillo-Fagundes *et al.*, In press). Il est également possible d’utiliser des lignées cellulaires placentaires, dont les cellules BeWo (cellules provenant d’un choriocarcinome humain) qui possèdent le phénotype villeux du cytotrophoblaste (Heaton et al., 2008, Sullivan, 2004), afin d’étudier le fonctionnement placentaire (Handschuh et al., 2007, Heaton et al., 2008, Lanoix et al., 2008, Moreau et al., 2001, Oufkir et al., 2010, Sanderson et al., 2001, Zygmunt et al., 1998).

Les moules et le placenta offrent également la possibilité de tenir compte de la différence entre les sexes, ce qui est souvent négligé dans les études toxicologiques (Mergler, 2012, Ritz *et al.*,

2014). Les moules sont dioïques et ses systèmes physiologiques peuvent dépendre du sexe (Mills *et al.*, 2003). Par exemple, l'expression de l'ARNm codant pour la cyclooxygénase (*COX)* est plus élevée chez les moules mâles que chez les femelles (Cubero-Leon *et al.*, 2010). Les paramètres immunitaires peuvent également varier selon le sexe, étant donné que le pourcentage d'hémocytes fluctuent de manière sexe-spécifique (Dang *et al.*, 2012). De plus, l'accumulation des contaminants dans les moules peut se produire différemment entre les mâles et les femelles (Richir *et al.*, 2014). La réponse à la contamination environnementale peut donc être différente lorsque le sexe est pris en compte (Aarab *et al.*, 2006, Cubero-Leon *et al.*, 2010). Il en va de même pour le placenta qui possède le même sexe que le fœtus, puisqu'il est issu des cellules embryonnaires. Il a été montré que l'expression des gènes dans le placenta et les fonctions placentaires peuvent varier selon le sexe du fœtus (Cleal *et al.*, 2010, Clifton, 2010), suggérant ainsi des réponses différentes vis-à-vis d'une exposition aux contaminants. Par exemple, une exposition du placenta au Cd cause une méthylation de l'ADN de manière sexe-spécifique (Mohanty *et al.*, 2015).

1.3.1. Placenta

Afin de nourrir et oxygéner l'embryon, les nutriments essentiels de la mère doivent obligatoirement traverser la barrière placentaire (Knipp *et al.*, 1999). Le placenta, constitué de tissus embryonnaires et maternels, joue le rôle de rein, de poumon, d'intestin et protège le fœtus du système immunitaire maternel. En raison de ses fonctions métaboliques et endocrines, le placenta est indispensable au maintien de la gestation, ainsi qu'à la croissance et au développement fœtal (Alsat *et al.*, 1998, Alsat *et al.*, 1999, Bauer *et al.*, 1998). Une altération des fonctions placentaires peut entraîner des pathologies de grossesse, telles que la prééclampsie (hypertension et protéinurie) et le diabète gestationnel, et induit également des complications néonatales, pédiatriques et parfois même des maladies métaboliques à l'âge adulte (Malassiné *et al.*, 2000, Redman *et al.*, 2005, Shapiro *et al.*, 2015, Tsatsaris *et al.*, 2008). Dans ce contexte, les études des mécanismes cellulaires et moléculaires du placenta semblent indispensables afin de mieux comprendre les complications de grossesse et leurs conséquences sur le développement fœtal (Alsat et al., 1998).

L'humain possède un placenta hémomonochorial complexe et unique, c'est-à-dire que le sang maternel est en contact avec le trophoblaste du placenta sans jamais entrer en contact avec le sang fœtal (Malassiné *et al.*, 2000, Marieb, 2004, Stulc, 1997). L'unité fonctionnelle permettant les échanges de nutriments entre la mère et le fœtus est la villosité choriale (Fig. 1.5) (Goyer,

1990, Goyer, 1991, Lafond *et al.*, 2004). Cette dernière est constituée de quatre types différents de cellules : l'endothélium des capillaires fœtaux, le mésenchyme, les cytotrophoblastes villeux et le syncytiotrophoblaste, issu de la fusion des cytotrophoblastes (Fig. 1.5) (Alsat *et al.*, 1998, Goyer, 1990, Goyer, 1991, Lafond *et al.*, 2004). La villosité choriale est également appelée barrière placentaire, car elle permet de filtrer les éléments du sang maternel protégeant ainsi en partie le fœtus (Gundacker *et al.*, 2012, Myren *et al.*, 2007).

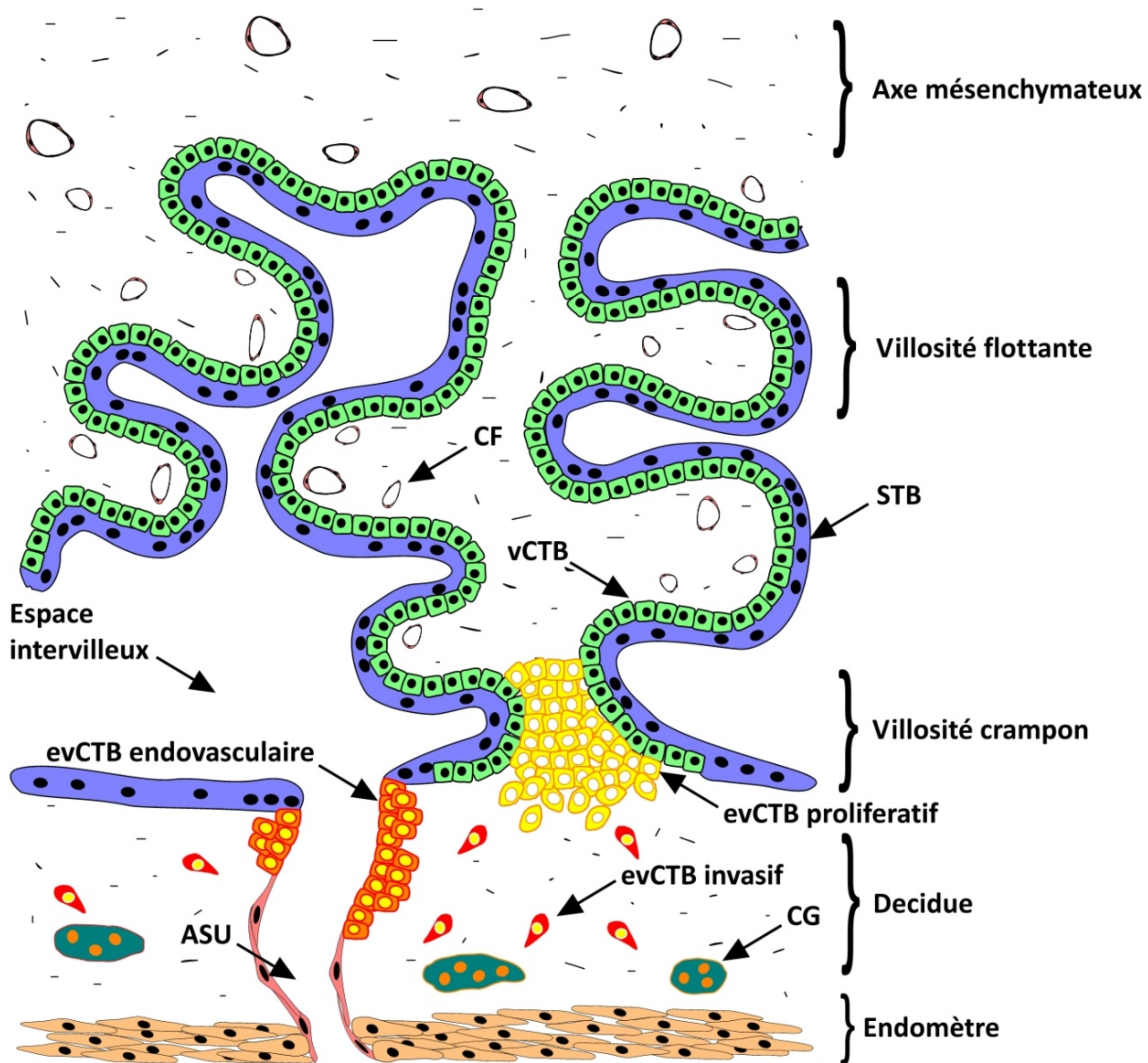

Figure 1.5 : Structure du placenta
Par l'artère spiralée utérine (ASU), le sang maternel envahit l'espace intervilleux où il entre en contact avec la villosité flottante. Le transport des nutriments s'effectue via la barrière placentaire constituée de 4 couches cellulaires : le syncytiotrophoblaste (STB), le cytotrophoblaste villeux (vCTB), le mésenchyme et les capillaires fœtaux (CF).
Cytotrophoblaste extravilleux (evCTB), Cellule géante (CG)
Figure offerte © D Lanoix

1.3.2. Moule

La moule bleue (*Mytilus edulis*) est un mollusque bivalve dioïque vivant sur les rivages rocailleux le long des côtes marines dans des moulières. On la retrouve dans la plupart des eaux polaires et tempérées. Elle se nourrit par filtration des particules en suspension dans l'eau, principalement de cellules phytoplanctoniques (Pêches et Océans Canada, 2003).

Au Canada, la mytiliculture est la deuxième culture aquacole la plus importante, derrière celle du saumon. Elle représente des revenus de plus de 45 millions de dollars, soit 54 % de la production de mollusques et 6 % de la production aquacole (Pêches et Océans Canada, 2015).

Pour se reproduire, la moule suit un cycle axé sur les saisons, soit la température de l'eau et la quantité de nourriture disponible (Fig. 1.6) (Lubet, 1959, Seed, 1976). Le cycle se décompose en plusieurs stades de reproduction qui débute par la gamétogénèse. Les ovocytes ou les spermatozoïdes produits sont emmagasinés dans les vésicules du manteau jusqu'au stade préponte. Les vésicules sont alors remplies de gamètes (Fig. 1.6A). Au moment de la ponte, on constate une augmentation des concentrations de 5-HT, accompagnée par une augmentation de l'activité de la cyclooxygénase (COX) et d'une diminution des concentrations de dopamine (Fong *et al.*, 1993a, Martínez *et al.*, 2000, Matsutani *et al.*, 1987). On constate également une diminution de la capacité phagocytaire des hémocytes des bivalves pendant la ponte associée à une diminution des réserves énergétiques (Cartier *et al.*, 2004, Fraser *et al.*, 2014, Fraser *et al.*, 2013, Li *et al.*, 2007, Li *et al.*, 2009, Li *et al.*, 2010). Cette diminution de leur système immunitaire peut rendre la moule plus vulnérable à la contamination environnementale (Annexe 3).

La ponte se produit, au moins, à deux reprises durant l'année selon la température de l'eau et de la quantité de nourritures capturées. Dans l'est du Canada, la ponte se produit généralement autour de début juin et de fin octobre (Cartier *et al.*, 2004, Lubet, 1959). L'émission des gamètes se produit par mouvement des cils provoquant des nuages blancs et orangés dans l'eau où la fécondation a lieu (Lubet, 1959). Par la suite, les moules entrent dans la dernière phase du cycle en procédant à une réabsorption des gamètes non émis (Fig. 1.6D) et à la restructuration de son manteau (Fig. 1.6E). Pendant ce stade de repos sexuel, aucun gamète ne peut être observé. Ainsi, lors de ces stades, les méthodes de sexage basé sur l'observation des gamètes ne semblent pas adéquates (Petes *et al.*, 2008, Sedik *et al.*, 2010).

L'accumulation des contaminants environnementaux dans les moules est entre autres, influencée par le sexe (Richir *et al.*, 2014, Sokolowski *et al.*, 2004). De plus, les systèmes

biologiques peuvent naturellement varier entre les mâles et les femelles (Cubero-Leon *et al.*, 2010, Dang *et al.*, 2012). Il semble donc important d'inclure le stade du cycle de reproduction et le sexe dans les études écotoxicologiques sur les bivalves.

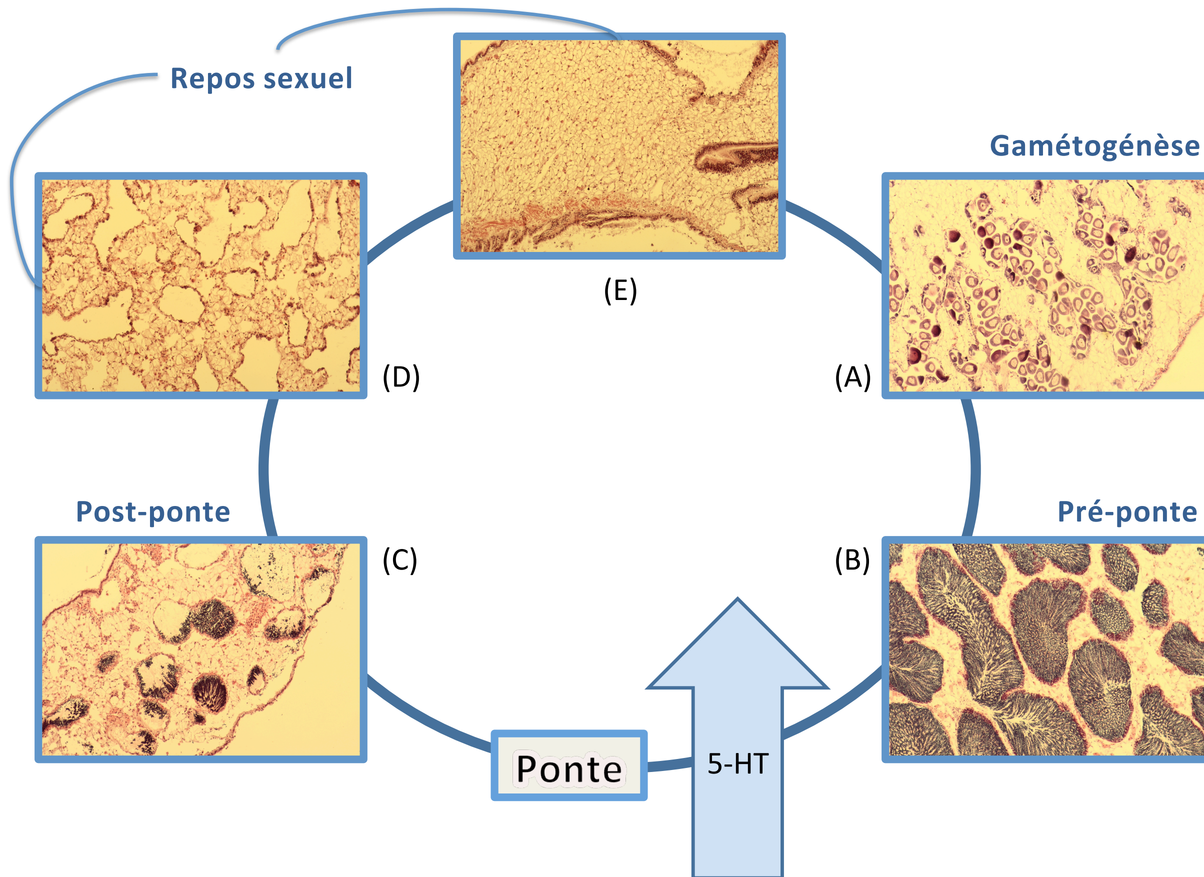

Figure 1.6: Cycle de reproduction de *Mytilus edulis*
Le cycle débute par la gamétogénèse (A) pendant laquelle les vésicules du manteau emmagasinent les ovocytes (A) ou les spermatozoïdes (B). En période pré-ponte (B), ces véhicules sont pleins. Sous l'action, entre autre, de la 5-HT, les gamètes sont émis dans l'eau par un mouvement des cils. Ce processus vide majoritairement les vésicules (C). Ce stade est suivi d'une période de réabsorption des gamètes (D) et de restructuration du manteau (E) correspondant à la période de repos sexuel. © M Fraser

1.4. Hypothèse et objectifs

La 5-HT est impliquée dans la régulation de la reproduction humaine et des mollusques. Chez l'humain, la 5-HT maternelle et placentaire est impliquée dans le bon déroulement de la grossesse et dans le développement du placenta et de l'embryon et le fœtus. Chez la moule, la 5-HT permet la gamétogenèse et induit la ponte. La moule et le placenta sont exposés à la contamination environnementale par les métaux. Les métaux, tels que le Mn, le Pb et le Cd, perturbent le système 5-HT au niveau du système nerveux de divers organismes. De plus, l'effet des métaux et l'expression ou la signalisation sérotoninergique varient selon le sexe. Par contre, à ce jour, les effets des métaux sur le système 5-HT de la moule bleue et du trophoblaste humain demeurent peu ou pas étudiés.

L'hypothèse de recherche de ce projet doctoral est que de faibles concentrations de Mn, Pb et Cd altèrent, de manière sexe-spécifique, le système 5-HT, chez la moule bleue et le trophoblaste humain. L'objectif général est d'étudier l'effet des métaux sur le système sérotonine dans les moules (*in vivo)* et le trophoblaste humain (*in vitro*), deux modèles non invasifs et accessibles.

Les objectifs spécifiques sont :

1) Déterminer un protocole efficace de sexage des moules. La littérature propose différentes méthodes d'analyses du sexe qui peuvent varier en efficacité selon le stade du cycle de reproduction et qui sont parfois mal appliquées. Aucune étude n'a comparé l'efficience des méthodes de détermination du sexe existantes et ne suggère de protocole de sexage clair et efficace. Étant donné que la 5-HT est impliquée dans les fonctions reproductrices des moules et que le système 5-HT et l'effet des métaux peuvent varier selon le sexe, il est important, pour l'étude du système de la 5-HT chez les moules, qu'un protocole soit clairement établit.

2) Déterminer l'effet d'une exposition à long terme du Mn, Pb et Cd sur l'expression du SERT, les concentrations de 5-HT et l'activité de la MAO dans la moule bleue et si les effets observés varient selon le sexe.

3) Déterminer l'effet d'une exposition au Mn et Pb sur le système sérotonine du trophoblaste placentaire humain.

3.1) Dans les cellules BeWo, un modèle *in vitro du trophoblaste villeux*, évaluer l'effet du Mn et Pb sur la concentration de 5-HT, l'activité de la MAO, l'expression et l'activité du SERT et l'expression du récepteur $5HT_{2A}$;

3.2) Évaluer l’effet du Mn et du Pb sur l’expression du SERT et du récepteur $5HT_{2A}$ dans les placentas d’une cohorte de femmes enceintes.

CHAPITRE 2 :
SEX DETERMINATION IN BLUE MUSSELS: WHICH METHOD TO CHOOSE?

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Contribution de l'étudiant

L'étudiant a participé à l'élaboration de l'étude, réalisé toutes les expériences et analysé les résultats dans cet article. Il a également rédigé l'article, participé au choix du journal de publication et effectué les corrections des « Reviewers ».

Résumé de l’article en français

Les méthodes de sexage des moules bleues sont principalement basées sur la présence ou l'absence de gamètes et ne prennent pas en compte les stade du cycle de reproduction. Les effets d’une exposition environnementale peuvent être influencée par le sexe de la moule. Le but de cette étude est donc de déterminer un protocole efficace de détermination du sexe, tout en tenant compte des stades du cycle de reproduction. Huit méthodes de sexage de moules ont été comparées. Cette étude démontre que la première étape pour sexer les moules bleues devrait être la détermination du stade de reproduction, ce qui peut être fait par l'histologie du manteau. Pendant la gamétogenèse, l’histologie permet de différencier les mâles des femelles par l'observation des gamètes. En revanche, lorsque les moules sont en repos sexuel, la seule méthode qui devrait être utilisée est celle des gènes spécifiques au sexe

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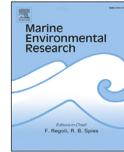

Sex determination in blue mussels: Which method to choose?

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ABSTRACT

Sexing methods of blue mussels are mostly based on the presence or absence of gametes, and do not take into account reproductive cycle stages. Exposure effects can be affected by the sex of mussels, thus the aim of this study is to determine an efficient sex determination protocol taking into account the reproductive cycle stage. Eight mussel sexing methods were compared. This study demonstrates that the first step in discerning sex in blue mussels should be assessing the reproductive stage, which can be done by mantle histology. During gametogenesis, histology allows the differentiation of males from females by the observation of gametes. However, when mussels are in sexual rest, the only method that should be used is the sex-specific gene method.

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1. Introduction

Not taking into account the sex differences in environmental and toxicological studies could lead to misinterpretation of results (Mergler, 2012; Ritz et al., 2014; Weiss, 2011). In bivalves, contaminants can bioaccumulate differently between sexes (Richir and Gobert, 2014; Sokolowski et al., 2004), and some biological functions differ between male and female (Banni et al., 2011; Brown et al., 2006). For example, the serotonin system, involved in sexual differentiation, gametogenesis and spawning (Cubero-Leon et al., 2010; Fong et al., 2003; Gagné and Blaise, 2003; Gibbons and Castagna, 1984), can be altered by contaminants (Almeida et al., 2003; Cubero-Leon et al., 2010; Salanki and Hiripi, 1990). Also, Dang et al. (2012) demonstrated that the percentage of hemocyte cell types fluctuates between sexes in *Saccostrea glomerata* and *Pinctada fucata*, especially for small agranulocytes. In *Mytilus edulis*, cyclooxygenase (COX) expression is higher in male gonads compared to female gonads (Cubero-Leon et al., 2010). Thus, the biological response pattern can diverge between sexes when mussels are exposed to xenobiotics such as endogenous estradiol which affects the COX expression in a sex-specific manner (Cubero-Leon et al., 2010).

Although there are a few studies that include bivalve sex in their analyses (Brown et al., 2006; Cappello et al., 2015; Falfushynska et al., 2013; Lazzara et al., 2012; Ruiz et al., 2011), it is still often neglected (Chandurvelan et al., 2012; Fraser et al., 2014; Klouche et al., 2015; Martinez Bueno et al., 2014). Several methods exist to determine mussel sexes (Hines et al., 2007; Jabbar and Davies, 1987; Mikhailov et al., 1995; Ruiz et al., 2011; Sedik et al., 2010). Most of them are based on the presence or absence of gametes in the mantle. This tissue is the main site of gonad development and stores gametes until spawning (Mikhailov et al., 1995). The mussel reproductive cycle is divided coarsely in four stages in which gametes are not always noticeable, especially in late post-spawning and sexual rest (Lubet, 1959; Ruiz et al., 2011; Seed, 1976). Briefly, the cycle starts by a stage without gametes where a rapid regeneration of the mantle takes place (post-spawning resting stage; stage 0). It is followed by the gamete formation (stage I). In the ripe stage (stage II), the storage vesicles abound in spermatozoon or oocytes: spawning is imminent. In the spawning and post-

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spawning stage (stage III), gametes are emitted in the water. This is followed by the degradation and reabsorption of the remaining gametes until the cycle starts over (Lubet, 1959; Ruiz et al., 2011; Seed, 1976). Thus, sex determination methods based on gametes are not effective and cannot be used at every reproductive cycle stages of mussels (Petes et al., 2008; Sedik et al., 2010). The aim of this study is to compare eight mussel sex determination methods in order to establish an efficient sex determination protocol that includes the reproductive cycle stage.

2. Materials and methods

2.1. Mussels handling

Blue mussels (>90% *Mytilus edulis*, and <10% *Mytilus trossulus* and hybrid) were collected from the *Baie de Plaisance* located in the *Îles de la Madeleine* region (47°29′N, 61°87′W) in early June 2013 by the mussel farm “La moule du large”. Since mussels of this region are known to spawn around this period, individual mussels will be at slightly different stages of the spawning process, but the population would be regarded as not having fully spawned or to have entered into a stage of atresia (Cartier et al., 2004). Mussels were placed in an aerated tank filled with artificial seawater (Instant Ocean®, Reef Crystal, Cincinnati, OH, USA) (15 °C, salinity 31 ± 1 PSU) for 28 days, then sacrificed, and the mantle dissected on ice. Spawning was not observed during the acclimation period.

Smears of the mantle were collected on microscope slides. A small piece of the mantle (3 mm^2) was collected for histology. The rest of the tissue was homogenized with a Teflon pestle tissue grinder in a conservation buffer (Hepes-NaOH buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM dithiothreitol and 1 μg/mL aprotinin) as described by Gagné et al. (2011c). Proteins in the homogenate were dosed with the Pierce bicinchoninic acid assay (BCA) according to the manufacturer's instructions (Pierce Biotechnology, CA). All samples were kept at −80 °C until analysis.

2.2. Sex determination analysis

All the sexing methods were applied on the 120 mussels to save time and cost. Two experimenters were assigned to establish mussels' sexes using random and blinded ID to decrease subjectivity. Each experimenter had to establish the sex of each mussel twice.

2.2.1. Mantle color

Sex was determined according to color of the mantle: creamy white for males and orange for females (Mikhailov et al., 1995).

2.2.2. Fresh smear

Two singular slide smears of the mantle were made on a microscope slide and immediately observed by optical microscopy at a magnification of 10× in order to observe the presence of moving spermatozoon to determine the sex of the mussel (Gagné et al., 2001). Mussels were considered female when spermatozoon was absent, even though this could cause bias when mussels are in resting stage.

2.2.3. Stained smear

Slides used for fresh smear observations (section 2.2.2) were dried at room temperature overnight and fixed and stained with the Kwik Diff Kit (Thermo Fisher Scientific, MA). The presence or absence of spermatozoon (purple dots) was observed by optical microscopy at a magnification of 10× to establish the sex of the mussel. Mussels were considered female when spermatozoon was absent.

2.2.4. Histology

Mantle samples (3 mm^2) were fixed in Bouin solution (Sigma-Aldrich, ON, Canada) for 24 h, dehydrated in a series of ethanol dilutions and embedded in paraffin. Six sections of 5 μm thickness were stained with Harris hematoxylin and eosin solutions (Thermo Fisher Scientific, MA). The reproductive cycle stage and the sex of each mussel, when gametes (spermatozoa and ova) could be observed, were determined by microscopy at a magnification of 10× according to Lubet (1959), Seed (1976) and Ruiz et al. (2011).

2.2.5. Chemical coloration

The chemical method described by Jabbar and Davies (1987) was used with minor modifications. Briefly, 75 μL of the homogenized tissue was mixed with 1 mL of 20% trichloroacetic acid (TCA) and 0.25 μL of 0.75% thiobarbituric acid (TBA) in a 1.5 mL tube. Mixtures were heated during 20 min at 95 °C. According to the color of the resultant solution, a sex was associated: yellow for males and pink for females.

2.2.6. Spectrophotometric analysis

The solutions obtained in 2.2.5 were centrifuged to eliminate tissue suspensions. A volume of 200 μL of each solution was placed in a clear 96 well plate and scanned over the 430–600 nm range with a spectrophotometer (Spectra Max M5, Molecular devices). A peak of absorbance at 460 nm reflects the relative abundance of DNA and oxidizable lipids (Hines et al., 2007; Jabbar and Davies, 1987). This is associated with male mussels.

2.2.7. Male associated polypeptide (MAP39)

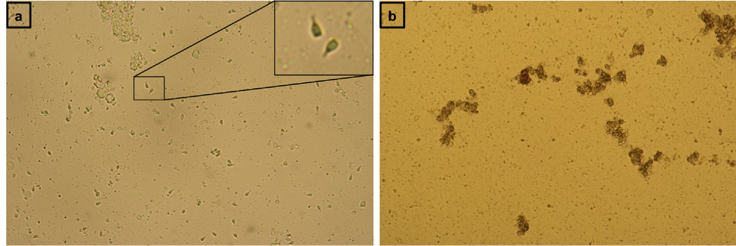
The male associated polypeptide (MAP39) was found in the mantle of *Mytilus galloprovincialis* lmk (Mikhailov et al., 1995). Briefly, proteins were extracted from the mantle homogenate solution with radioimmunoprecipitation buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA) containing protease and phosphatase inhibitors cocktails (Pierce, ThermoScientific). After sonication, solutions were centrifuged (14,000× *g*) at 4 °C during 10 min for protein extraction. Protein concentrations were determined in the supernatant with the BCA kit using the manufacturer's instructions (Pierce Biotechnology, Rockford, CA). Forty micrograms of proteins were separated on 10% SDS-PAGE gel with a running buffer (10% Sodium dodecyl sulfate; *7.2% glycine and 1.5% Tris*) and stained with Coomassie blue. The

Table 1
PCR conditions for the sex-specific gene method.

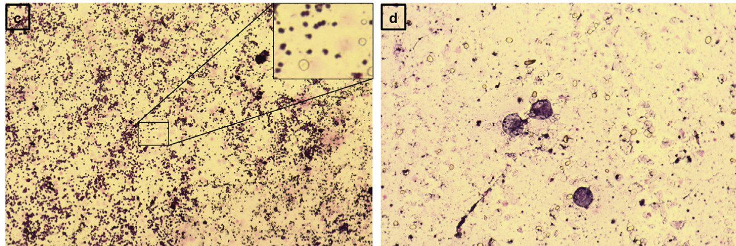
Gene (genbank)	Sequence		Annealing	Product (bp)	Reference
verl (FM995161.1)	F	5′-CTGCAATGGTTTTGGTTGTG-3′	50 °C	350	Hines et al. (2007)
	R	5′-CCGAAGGAAATGGAACTGAA-3′			
Vcl (FM995162.1)	F	5′-TTGCGTTTCACATGGTTGAT-3′	55 °C	250	Sedik et al. (2010)
	R	5′-AGAGCTGTTTTGGCCACAGT-3′			

verl: vitelline envelope receptor for lysine; *vcl*: vitelline coat lysine.
F: Forward; R: Reverse.

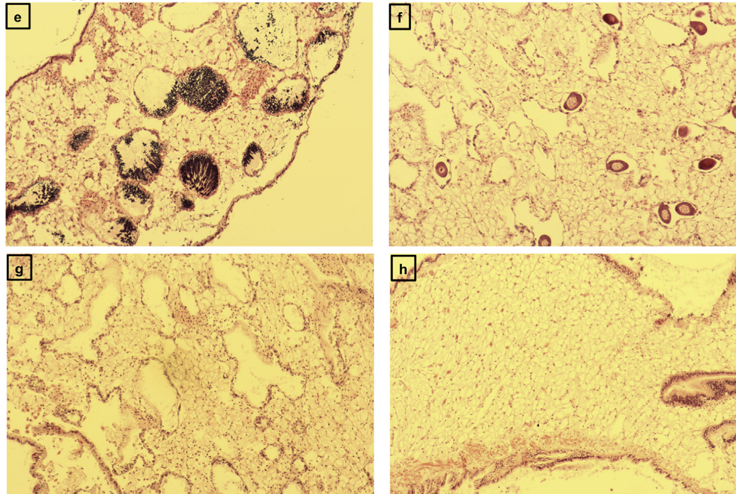
Fresh smears



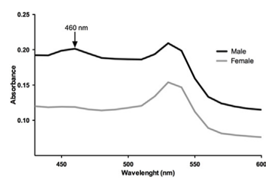
Colored smears

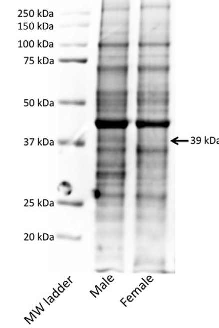


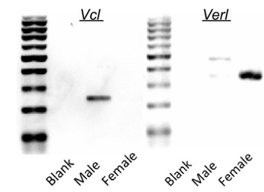
Histology



i Spectrophotometry **j MAP39** **k Sex-specific mRNA expression**







Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standards (BioRad) was used. The colorimetric pictures were made with the Chemidoc MP imaging system (Bio-Rad, ON, Canada). Using Image Lab 4.1 software (Bio-Rad, ON, Canada), molecular weights of proteins were determined in order to find clear and thick 39 kDa bands, which is associated with males. It should be noted that the 39 kDa band could be composed of multiple peptide types and not only MAP39.

2.2.8. Sex-specific mRNA expression

Vitelline envelope receptor for lysine (*Verl*) and vitelline coat lysine (*Vcl*) are specific genes for female and male respectively (Hines et al., 2007; Sedik et al., 2010). Total RNA was extracted from 250 μL of the mantle homogenate with the Aurum total RNA extraction kit (Bio-Rad) following the manufacturer's protocol. Briefly, homogenate was mixed with RNA extraction kit's lysis solution (Bio-Rad) and homogenized by a QIAShredder (Qiagen, ON, Canada). Genomic DNA was digested by DNase for 15 min. RNA was eluted in 40 μL of the elution buffer included in the extraction kit. RNA concentration and purity were measured by spectrophotometry (nanoDrop 1000 spectrophotometer, Thermo Fisher Scientific) and then stored at −80 °C until analysis. Only RNA with an OD 260/280 ratio of 1.8–2.0 was used. Reverse transcription reaction was performed with the iScript cDNA synthesis kit (Bio-Rad) following the manufacturer's instructions. PCR reactions were performed according to the PCR Taq DNA Polymerase Kit protocol (Qiagen) and using the T3000 thermocycler (Biometra, Göttingen, Germany). Primer sequences and molecular weight are presented in Table 1. PCR products were migrated on 2% agarose gel containing 5 μL ethidium bromide. Gel Doc™ EZ System (Bio-Rad) was used to take the fluorescent pictures. Images were processed with the Image Lab 4.1 software (Bio-Rad).

2.3. Statistical analysis

The sex-specific gene method, based on the presence of sex-specific transcripts of sperm (*Vcl*) and oocyte (*Verl*), is considered highly accurate at all reproductive cycle stages (Hines et al., 2007; Sedik et al., 2010) and was used as a gold standard method. Receiver Operating Characteristic (ROC) Curves were applied in order to determine the performance of each method (Park et al., 2004). This analysis also determined the sensitivity and false positive (100-specificity) of the methods for each mussel sexed. Kappa (κ) analyses were performed to determine an agreement level between a method and the gold standard (Viera and Garrett, 2005). Finally, chi-square tests were used to compare results. $P < 0.05$ was considered statistically significant.

3. Results

Fig. 1 shows representative results for each method used to determine the sex of the mussels, except those based on color observation. The fresh smear method shows living spermatozoon in male mussels (Fig. 1a) compared to females (Fig. 1b). After staining the fresh smear slides, the spermatozoon appear as little purple dots for males (Fig. 1c), but not for females (Fig. 1d). Mussel reproductive cycle stages are shown in Fig. 1e–h: post-spawn (Fig. 1e and f: Stage IIIB), late post-spawn (Fig. 1g: Stage IIID) and sexual rest (Fig. 1h: Stage 0). When spermatozoon (Fig. 1e) or oocytes (Fig. 1f) are in the mantle vesicles, sex determination is simple, whereas in the absence of gametes, it is not possible to determine the sex (Fig. 1g and h).

Blue mussels (n = 120) were collected from the mussel farm in early June, a period when mussels are known to spawn (Cartier et al., 2004). Histology confirms that all mussels were in post-spawn (with or without residual gametes) or sexual rest stage (Fig. 1e–h). Only 25.8% (n = 31) of mussels contained gametes in their mantle (data not shown). The small absorbance peak observed at 460 nm in the black curve of Fig. 1i and the darker band at 39 kDa, associated with MAP39 (Fig. 1j), indicate male mussels. Fig. 1k shows migrated RT-PCR product for sex-specific genes *Vcl* (male) and *Verl* (female).

The sex-specific genes method allowed to determine the sex of each muscle without ambiguity (example: Fig. 2a; data not shown for the 120 mussels) and has been used as the gold standard method to determine the validity of the other methods. The validity of each method without taking into account the reproductive cycle stage did not exceed 73.6% (fresh smear) and can be as low as 25.8% for histology (Fig. 2a). However, if the analysis is done on mussels containing gametes only (n = 31) histology reached a perfect score of 100% (Fig. 2b).

Fresh and stained smears, spectrophotometry, and histology methods show higher matching percentage (>68.1%; Fig. 2b). When comparing the smear methods, staining of the fresh smears significantly increased the overall sexing performance to 100% ($\chi^2 = 4.28$; $p = 0.039$; Fig. 2b). The mantle color and MAP39 demonstrated a lower sexing performance of 29% and 45.6% respectively, and the κ values indicated that neither of the methods is better than luck to determine mussel sexes correctly (Fig. 2b). All methods showed similar sensitivity for both sexes when considering the reproductive cycle stage with gametes ($\chi^2 < 3.84$, $p > 0.05$), except for the spectrophotometry methods which demonstrated significantly less sensitivity in order to correctly assign sex to males compared to females ($\chi^2 = 4.91$, $p = 0.027$; Fig. 3a(i)). Methods showed less than 35.3% of false positives except for mantle colors (71.4% for males and 70.4% for females) and MAP39 (58.8% for females) (Fig. 3a(ii)). This is in contradiction with the results obtained when mussels are in a reproductive cycle stage without gametes (Fig. 2c). Histology, which is based on the observation of the gametes, showed a score of sexing performance of 0% when mussels are in reproductive cycle stages without gametes (Fig. 2c). All other methods demonstrated an overall sexing performance <70% with fair to poor agreement with the PCR gold standard method (Fig. 2c). Sensitivity for females showed a sexing performance >70% for fresh and stained smears, and reached a perfect score with spectrophotometry methods (Fig. 3b(i)), whereas sensitivity for male was significantly lower ($\chi^2 > 3.84$, $P < 0.05$; Fig. 3b(i)). Results of sensitivity were not different between males and females for mantle color and MAP39 ($\chi^2 = 0.160$ and $\chi^2 = 0.003$; $P < 0.05$; Fig. 3b(i)). False positive analysis demonstrated that methods could be highly sensitive for females without being specific (Fig. 3b(ii)).

Fig. 1. Representative results of each method used to determine mussel sex. The spermatozoon presence (**a**, **c**; boxes represent a zoom on spermatozoon) or absence (**b**, **d**) on fresh smears and colored smear slides served to distinguish between male and female. Histology (**e-h**) served to determine the sex (**e**: male; **f**: female) and also the reproductive cycle stages (**e, f**: post-spawn (stage III B); **g**: late post-spawn (stage III D); **h**: sexual rest (stage 0)). The chemistry method using spectrophotometry (**i**) showed an absorbance peak at 460 nm for male mussels. Male-associated polypeptide (MAP39), observed at 39 kDa on a polyacrylamide gel and colored by Coomassie blue, indicated a male mussel (**j**). RT-PCR sex-specific mRNA expression demonstrates the expression of vitelline envelope receptor for lysine (*Verl*) expressed in female and vitelline coat lysine (*Vcl*) expressed in male in the mantle (**k**). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

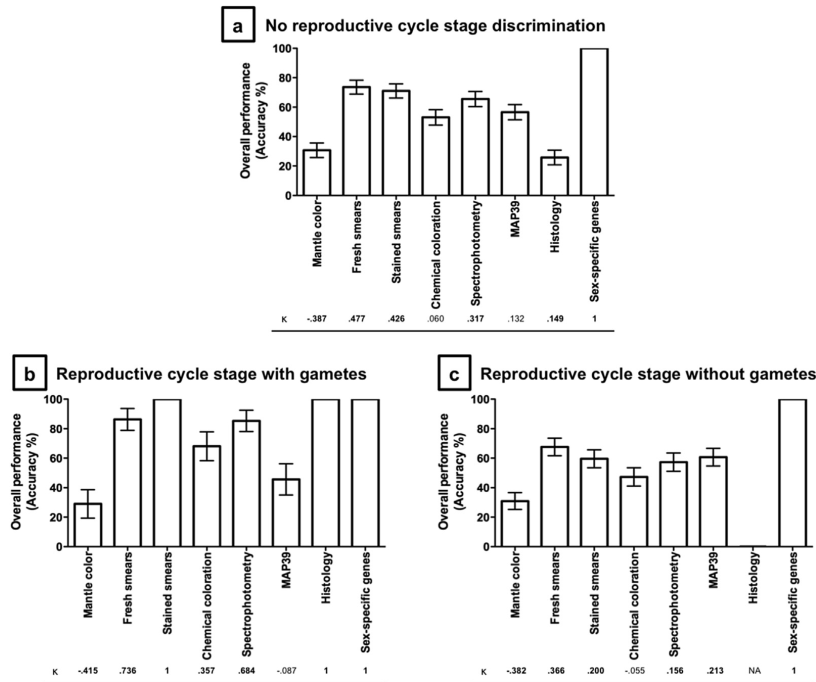


Fig. 2. Sexing performance of each method used to determine mussel sexes using sex-specific genes analysis as a "gold standard". The area under the curve of the ROC curve analysis was expressed in accuracy percentages for each method taking all reproductive cycle stages into account (**a**) or focusing on reproductive cycle stages with (**b**) or without (**c**) gametes. Error bars represent the standard deviation. Under each graph, Kappa (κ) statistic, the agreement level of each method with the sex-specific genes method, is presented. Statistical significant κ are in bold ($P < 0.05$).

4. Discussion

This study confirms the importance of knowing the mussel reproductive cycle stage in determining the sex correctly (Anantharaman and Craft, 2012; Hines et al., 2007; Mikhailov et al., 1995). Five out of 8 methods were unreliable indicators of mussel sex. When mussels are in a reproductive cycle stage containing gametes, histology and stained smears give the correct sex. Thus, the very first step in order to determine mussel sexes should be using the stained smear method since it allows to process a large number of samples in a short period of time. Nevertheless, if gametes (spermatozoa or ova) are not observed with the stained smear method, histology should always be made to ensure the reproductive cycle stage. If gametes are not observed in histologic analysis, the sex-specific gene method should be used. Thus, considering that nearly all the methods developed to determine the sex of mussels are based on spermatozoon presence in the mantle, this study shows that reproductive cycle stage should always be determined before sexing mussels. Moreover, the methods studied demonstrate that absence of spermatozoon caused interpretation bias in favor of the female, except for histology and PCR where specific identification of females was done. The sex-specific gene method, based on the presence of sex-specific transcripts of sperm (*Vcl*) and oocyte (*Verl*), is considered highly accurate (Hines et al., 2007; Sedik et al., 2010). The method was adequate for all reproductive cycle stages on the first try. The present study shows that the sex-specific genes method should be the only sexing method considered in reproductive cycle stages without gametes or when reproductive cycle stages are not known. This is particularly important since most of the studies on mussels in northern regions are conducted in warm seasons (to be able to reach mussels without ice constraints), a period when mussels are often in post-spawn reproductive stages (Cartier et al., 2004). It is to be noted that Anantharaman et al. demonstrated that mussel reproductive cycle stages and sex can be determined at the same time by RT-qPCR (quantitative PCR) (Anantharaman and Craft, 2012).

Histology analysis confirms that all studied mussels were in a post-spawn or sexual rest stage. Since most of the methods used in this study are based on the presence of spermatozoon, mussels can be wrongly sexed as a female in reproductive cycle stages without gametes. This bias is more important for the spectrophotometry method derived from Jabbar and Davies (1987). However, the methods used here differ from previous studies (Hines et al., 2007; Jabbar and Davies, 1987). In this study, the spectrophotometric analysis was realized on mantle homogenates instead of mantle tissues pieces as in Jabbar and Davies (1987) and Hines et al. (2007). Even though the coloration appeared as expected, the results obtained for the spectrophotometric analysis differ from those of Jabbar and Hines's papers. This may be caused by the omission of the butanol extraction in our protocol. Butanol extraction is used to concentrate proteins and DNA in the aqueous phase. This may result in removing parts of the matrices and concentrate molecules that are responsible for the missing or small peak we observed in our study. The peak at 530 nm observed for both sexes is not surprising. The reaction of TBA with malondialdehyde, a product formed during the degradation of lipids, produces a pink color that

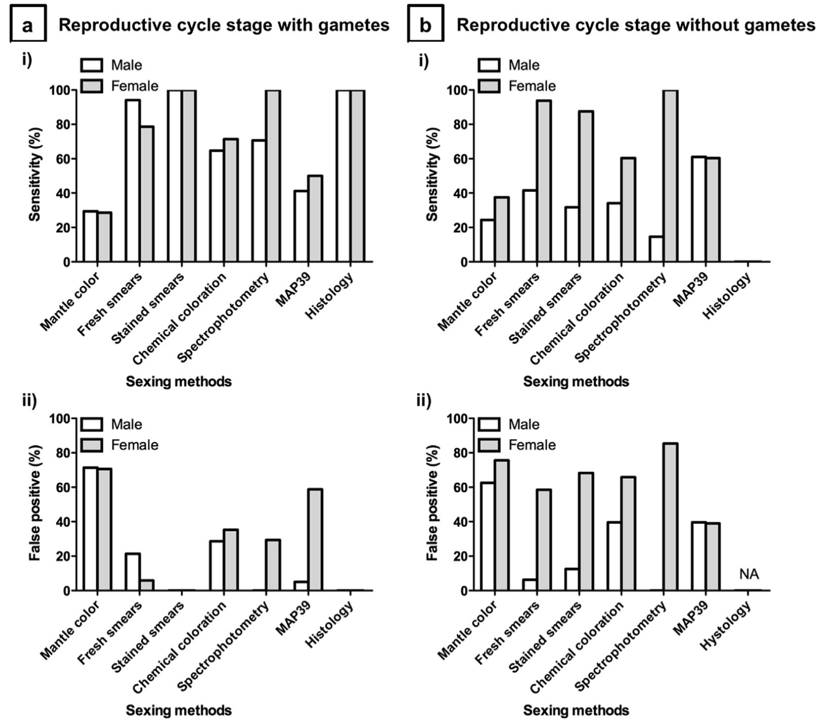


Fig. 3. Sensitivity (i) and false positive (ii) of each method used according to the mussel sex when compared to the sex-specific gene analysis. Although a method can reach great sensitivity, the specificity (represented by false positive) can be high and a bias can occur. Results were obtained with the ROC curve analysis and expressed in percentage focusing on reproductive cycle stages with (**a**) or without (**b**) gametes. NA – Non-applicable.

can be measured at 530–540 nm (Gagné et al., 2010; Jabbar and Davies, 1987; Wills, 1987). The butanol extraction realized by Jabbar and Davies before the absorbance screening, which was not done here, could explain why our results did not concur with previous results (Jabbar and Davies, 1987). Moreover, all mussels in our study were in a post spawned reproductive cycle stage. Jabbar and Davies (1987) and Hines et al. (2007) also demonstrated that the method was not reliable for mussels in that period. Nevertheless, in our study, when mussels had remaining gametes, the small peak observed at 460 nm associated with male mussels allowed to correctly sex 70.6% of males and 100% of females.

The lack of spermatozoon in the mantle can cause difficulties in interpretation when using color based methods, because of mantle color or chemistry color, and observation of the male associated polypeptide (MAP39). MAP39 is a major protein in gonad fluid and spawned sperm (Torrado et al., 2003). Mikhailov et al. (1995) showed that the expression of the protein follows the reproductive cycle and that females also express it to a smaller extent. That is why, in the context of the present study, females and males showed close overall performance percentage in reproductive cycle stages with or without gametes. The MAP39 expression observed by Coomassie coloration was not a good method for sexing in all reproductive cycle stages.

Traditionally, mussels were visually sexed by mantle color and this method is still used in recent studies (Mikhailov et al., 1995; Petes et al., 2008; Richir and Gobert, 2014). However, this method has proven to be inadequate in this study, as well as in other studies (Mikhailov et al., 1995; Petes et al., 2008). An orange mantle, identified as female, is mainly due to the high concentrations of carotenoid pigments. These concentrations change following the reproductive cycle stage (Campbell, 1969). Moreover, males can also display high concentrations of these pigments according to their position in the mussel bed, leaving room for subjective interpretation on color (Petes et al., 2008).

Methods based on observation of gametes should be 100% efficient at reproductive cycle stages with gametes, which is the case for stained smears and histology. However, fresh smears did not achieve the perfect score as expected. This can be due to the experimenter's subjectivity associated with problems distinguishing moving spermatozoon (fresh smear) or purple dots (colored smear). Thus, if spermatozoon were not moving or were not present on slides, mussels were considered female causing a bias in favor of females as confirmed by the high false positive percentage. Since all mussels were in post-spawn reproductive cycle stages, spermatozoon were in small amounts in the mantle. This can explain why the sensitivity of the methods did not achieve 100% even when spermatozoon can be seen in histology. Moreover, in reproduction cycle stage without gametes, the presence of spermatozoon (moving cells and purple dots) was associated with males, and the absence of spermatozoon in a mussel smear was associated with females. This explains why females had high false positive percentage compared to males, since male mussels did not have gametes in their mantle vesicles, as shown by histology. Thus, males should have obtained 0% sensitivity, instead of 41.5%. For

males correctly sexed, it is probably more about "luck" as the κ value demonstrates (κ = 0.366; $P < 0.05$). Experimenter subjectivity could also play a role. Indeed, smear methods, though they can be fast and easy ways to determine mussel sexes, are subject to misinterpretation and should only be used when the reproductive cycle stage is known. To decrease experimenters' subjectivity in some methods, results could be digitalized. For example, using a picture of the mantle, color could be expressed in RGB color codes, thus the sex could be determined by comparing the level of "red" to a threshold: more "red" would be associated with females. This kind of digitalization could also be applied to chemical coloration, spectrophotometric (presence/height of a peak) and MAP39 (presence/intensity of the band) methods.

When looking at the literature, inadequate methods were often used (Richir and Gobert, 2014). Other studies either omit to mention which method was used (Banni et al., 2011; Gonzalez-Rey and Bebianno, 2014; Gonzalez-Rey et al., 2014; Hazelton et al., 2013), do not identify the reproductive cycle stage when using a method that calls for it (Gagné et al., 2011c), or simply do not identify sex (Chandurvelan et al., 2012; Fraser et al., 2014; Klouche et al., 2015; Martinez Bueno et al., 2014). The present study, using traditional as well as new methods for mussel sexing, is the first to provide a clear protocol on how to correctly determine mussel sexes.

5. Conclusion

Mantle color, fresh smears, chemistry color, spectrophotometry and MAP39 methods are unreliable indicators of sex in mussels, although these methods are generally easier, less time-consuming and less expensive than others. Since reproductive cycle stages are regulated by external parameters such as temperature and availability of food (Cartier et al., 2004; Ruiz et al., 2011), mussel sex should always be determined first by histology, which also allows determination of the reproductive cycle stage. This is crucial since biological response to contaminants can differ between reproductive cycle stages (Cubero-Leon et al., 2010; Ruiz et al., 2011). If the mantle contains gametes, mussel sex is evident. On the other hand, if no gametes are observed, the sex-specific gene method should always be used to adequately determine mussel sexes (Anantharaman and Craft, 2012; Hines et al., 2007; Sedik et al., 2010). Finally, it is crucial to clearly mention the sexing method used in papers studying mussels.

Acknowledgments

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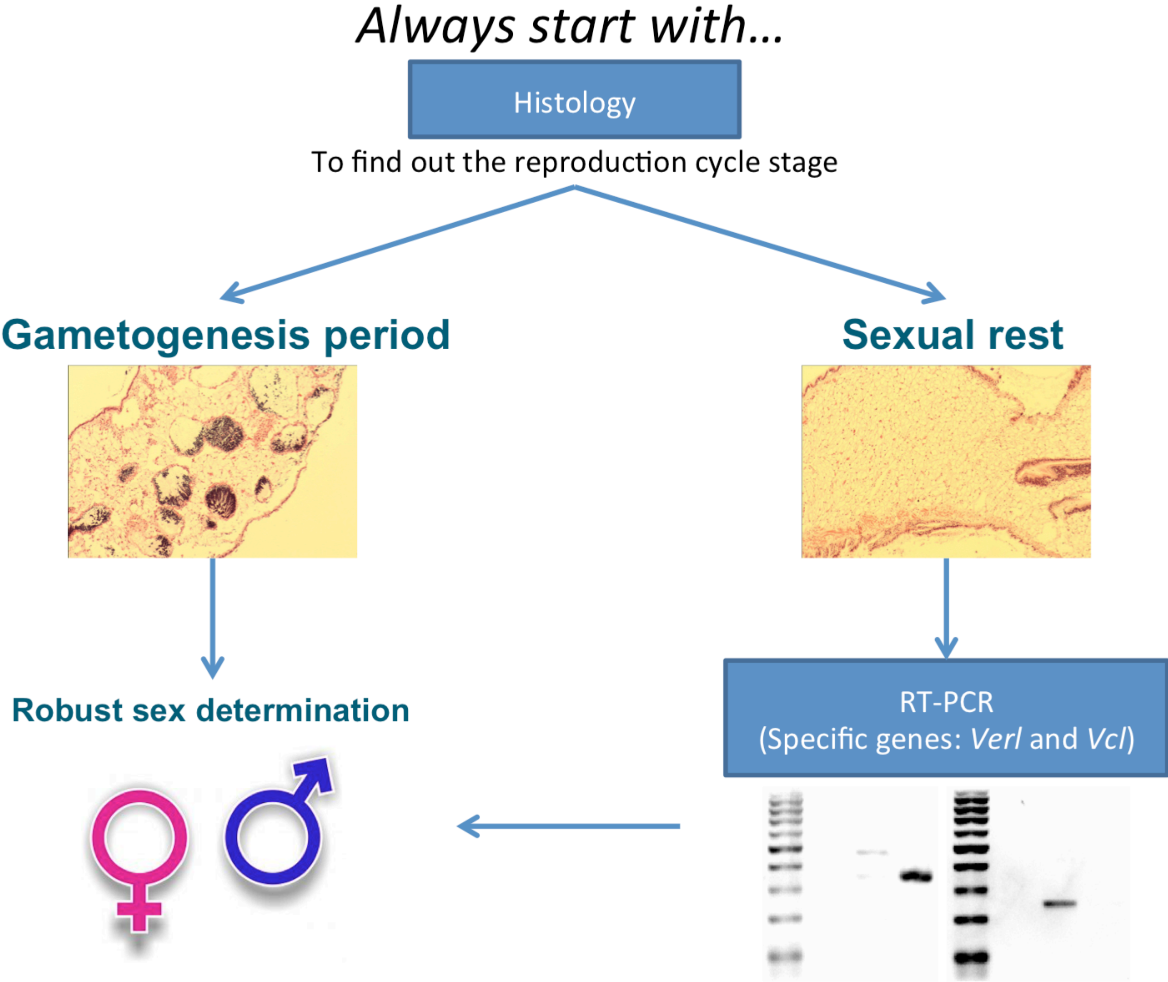
2.2. Graphical abstract



CHAPITRE 3 : EXPOSURE TO LOW ENVIRONMENTAL CONCENTRATIONS OF MANGANESE, LEAD, AND CADMIUM ALTERS THE SEROTONIN SYSTEM OF BLUE MUSSELS

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** Auteur-ressource*

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Contribution de l'étudiant

L'étudiant a participé à l'élaboration de l'étude, réalisé toutes les expériences et analysé les résultats dans cet article. Il a également rédigé l'article et participé au choix du journal de publication.

Résumé de l’article en français

La sérotonine, qui peut être affectée par les métaux, joue un rôle crucial dans la survie et la reproduction des moules. Cependant, les effets d’une exposition à des concentrations environnementales de métaux, tels que le manganèse (Mn), le plomb (Pb) et le cadmium (Cd), n’ont jamais été étudiés dans les moules bleues. Cette étude vise à déterminer les effets de l’exposition au Mn, Pb ou Cd sur les niveaux de sérotonine, l’activité de la monoamine-oxydase (MAO) et du transporteur de la sérotonine (SERT) dans les moules bleues (Mytilus edulis). Les moules ont été exposés *in vivo* à des concentrations croissantes de Mn (10 à 1000 nM), Pb (0,01 à 10 nM) et Cd (0,01 à 10 nM) pendant 28 jours. Les niveaux de sérotonine, l’activité de la MAO et l’expression de SERT ont été analysés dans le manteau des moules. L’expression de la protéine SERT est significativement diminuée, jusqu’à 81 %, par une exposition au Mn, au Pb et au Cd. L’activité de la MAO chez les moules femelles est presque 2 fois plus élevée que chez les mâles dans le bassin témoin. Dans les moules exposées à 0,1 nM de Pb, l’activité de la MAO est augmentée chez les moules mâles, alors qu’elle est diminuée chez les femelles. Une courbe dose-réponse non monotone, inversée selon le sexe, est observée pour l’activité de la MAO dans les moules exposées aux Cd. Cette étude montre que de faibles concentrations environnementales de Mn, Pb et Cd affectent le système de la sérotonine dans les moules bleues.

EXPOSURE TO LOW ENVIRONMENTAL CONCENTRATIONS OF MANGANESE, LEAD, AND CADMIUM ALTERS THE SEROTONIN SYSTEM OF BLUE MUSSELS

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3.1. Abstract

Serotonin, which can be affected by metals, plays a crucial role in mussel survival and reproduction. However, effects of environmental concentration of metals, such as manganese (Mn), lead (Pd), and cadmium (Cd), have never been studied in blue mussels. This study aims to determine the effects of exposure to Mn, Pb or Cd on serotonin levels, monoamine oxidase (MAO) activity, and serotonin transporter (SERT) in *Mytilus edulis*, blue mussels. Mussels were exposed *in vivo* to increasing doses of Mn (10–1000 nM), Pb (0.01-10 nM), or Cd (0.01-10 nM) for 28 days. Serotonin levels, MAO activity, and SERT expression were analyzed in mussel mantle. SERT protein expression is significantly decreased, up to 81%, by exposures to Mn, Pb, or Cd. MAO activity in females is almost 2-fold higher than males in none exposed control mussels. In mussels exposed to 0.1 nM of Pb, MAO activity is increased in males, whereas it is decreased in females. Inverted non-monotonic patterns of MAO activity is observed, according to sex, in mussels exposed to Cd. This study demonstrates that low environmental concentrations of Mn, Pb, and Cd affect the serotonin system in blue mussels.

Highlights

- MAO activity in blue mussel mantle is higher in females than males.
- Mantle MAO activity of blue mussels is affected differently between females and males exposed to Cd and Pb.
- Metals decreased significantly SERT protein expression, but not 5-HT level, in mussel mantle.

Keywords

Serotonin transporter, Reproduction, Monoamine oxidase, *Mytilus edulis*

3.2. Introduction

Mussels are ubiquitous, abundant, resistant to contamination, non-migrants and can bioaccumulate various contaminants, and thus widely used as bioindicators of metal pollution (Bourgoin, 1990, Cantillo, 1998, Chase *et al.*, 2001, Yusof *et al.*, 2004). Toxic and essential metals can have deleterious effects on physiological and immune systems of bivalves (Canesi *et al.*, 1998, Fraser *et al.*, 2014, Gagnaire *et al.*, 2004, Naimo, 1995, Rault *et al.*, 2013). Metals can also alter the serotonin (5-HT) system (Chand Basha *et al.*, 2014, Subhash *et al.*, 1991, Wu *et al.*, 2015). An acute exposure to lead (Pb), cadmium (Cd) or iron (Fe) decreased the 5-HT levels in brown mussels (*Perna perna*) (Almeida *et al.*, 2003). This is concordant with the results obtained by Salanki *et al.* (1990) who observed a decrease of 5-HT levels in freshwater mussel (*Anodonta cygnea*) ganglia exposed to Pb and Cd.

Serotonin is synthesized from L-tryptophan by a series of reactions rate-limited by the enzyme tryptophan hydroxylase (TPH) (Vrana *et al.*, 1993) and enter in the cell by its transporter (SERT) to mediate its action or to be degraded by monoamine oxidase (MAO) enzymes (Blakely *et al.*, 1991, Jonnakuty *et al.*, 2008). Serotonin system regulates mussel metabolism and is implicated in various reproductive processes such as sexual differentiation, gamete production and spawning (Aiello, 1965, Fong *et al.*, 2003, Gibbons *et al.*, 1984, Gies, 1986, Ram *et al.*, 1999). Thus, a disruption of the 5-HT system could alter mussel reproduction. However, metal effects on the 5-HT system in blue mussels *(Mytilus edulis)* have never been studied.

The aim of this study was to determine the effects of long-term exposure to low environmental concentrations of toxic metals, Pb and Cd, and an essential metal, Mn, on 5-HT levels, SERT expression and MAO activity. Sex can influence metal accumulation and biological activity, thus the effect sex differences of metal exposure on the 5-HT system in mussels were analyzed (Aarab *et al.*, 2006, Cubero-Leon *et al.*, 2010, Dang *et al.*, 2012, Mergler, 2012).

3.3. Materials and methods

3.3.1. Mussels handling and metal exposure

Blue mussels (*Mytilus edulis)* were collected from the *Baie de Plaisance* located in the *Îles de la Madeleine* Region, Québec, Canada (47°29'N, 61°87'W) in early June of 2013. Prior to the experimentation, the specimens were placed in a tank containing a polyethylene bag (Aquamerik, Inc., QC, Canada) filled with artificial seawater (Instant Ocean®, Reef Crystal, OH; 15°C, salinity 31-1psu) for 14 days and fed three times a week with phytoplankton (Phytoplex®, Kent Marine, WI). Water was continually aerated. Mussels of similar length and weight were then divided into 10 different 10 L aerated tanks (14 mussels per tank) and placed in a 20.2 ± 0.8°C room under photoperiod cycle of 12 h for 7 days of acclimatization. Mussels were not fed during acclimatization and experimentation. In the exposure tanks, $MnCl_2$, $PbCl_2$ or $CdCl_2$ at environmental concentrations (Mn: 10, 100 or 1000 nM; Pb and Cd: 0.01, 0.1 or 10 nM; (CCME, 2007, Fraser *et al.*, 2011, GDWQ, 2004)) were added to the artificial seawater, to simulate sub-chronic exposure for 28 days (long-term exposure). Water and the polyethylene bag of each tank, including the control tank (artificial seawater), were renewed every three days. Before and after water replacement, water samples were collected in acid-washed 15 mL tubes from each tank to analyze metal concentrations. Mussel survival and water temperature were monitored every day.

After the 28 days' exposure period, 12 mussels from each tank were individually weighed and measured (all mussels combined: 64 ± 4 mm and 26 ± 4 g) before dissection on ice of the mantle where the gametogenesis takes place. A small piece of the mantle (3 mm^2) was collected in order to establish the reproductive cycle stage by histology. The other part of the mantle was homogenized, on ice, with a Teflon pestle tissue grinder in a conservation buffer (Hepes-NaOH buffer pH 7.4 containing 100 mM NaCl, 0.1 mM dithiothreitol and 1 µg/mL aprotinin) as described by Gagné *et al.* (2011c). The cytoplasmic fraction (S15) was obtained from a sub-sample of the homogenate as described by Gagné *et al.* (2011b). Proteins in homogenates and S15 fractions were quantified with the Pierce bicinchoninic acid assay (BCA) according to the manufacturer's instructions (Pierce Biotechnology, Rockford, CA). All samples were kept at -80°C until analysis.

One mussel died during the seven days acclimatization period and five died during the experiment from five different exposure tanks, giving 99.3 and 96.4 % of survival rates, respectively (supplementary table S1).

3.3.2. Water Metal Concentrations

Metals concentrations (Mn, Pb, and Cd) in the seawater were determined using the Thermo Scientific iCAP-Q inductively coupled plasma mass spectrometer (ICP/MS; Bremen, Germany) interfaced with the ASX-520 auto-sampler from CETAC Technologies (Omaha, USA). Polyatomic interferences were minimized using the collision cell configuration of the instrument and with helium (He) as a collision gas and instrumental drift was corrected using an internal standard solution (Scandium (Sc) at 50 µg L^{-1}, yttrium (Y) at 15 µg L^{-1}, and indium (In), holmium (Ho), and terbium (Tb) at 5 µg L^{-1} – Inorganic Ventures, internal standard stock solution at 10,000 µg L^{-1}; Delta Scientific, Canada). Calibration standards (*Plasma* CAL, ICPMS verification standards at 10,000 µg L^{-1} from SCP Science, Canada) and internal standard solutions were prepared fresh daily by sequential dilutions of stock solutions. The measurement sequence included analysis of samples, sample replicates, method and instrumental blanks, calibration standards, and certified reference materials. Prior to analysis, all samples were diluted by a factor of 25 using 0.16 M nitric acid (HNO_3, Trace metal grade, Fisher Scientific) to reduce the sample matrix from initial salinity of 31 psu (3.1 %) to recommended levels of total dissolved solids inferior to 0.15% (Kutscher *et al.*, 2014). Measurements analyze were validated using the certified reference materials (CRMs): CASS-5 Nearshore Seawater - Reference Material for Trace Metals (NRC, Canada), ES-L-1 EnviroMAT Groundwater (SCP Science, Canada) and EP-L-1 EnvironMAT Drinking Water (SCP Science, Canada). As the concentrations for both Pb and Cd in the certified seawater CASS-5 were below quantification limits, the validity of our measurements in matrix-like samples using two house-made seawater (salinity of 31 psu) spiked solutions at concentrations of 0.5 and 5.0 µg L^{-1} (i.e. 9.1 and 91.0 nM for Mn, 4.5 and 44.5 nM for Cd, and 2.4 and 24.1 nM for Pb) was assessed. Average accuracy for all CRMs and spiked solutions were 87.6 ± 11.0% for Mn (n = 13), 98.7 ± 5.3 % for Cd (n = 6), and 92.6 ± 6.8 % for Pb (n = 6). For all three metals, the precision measured for CRMs was better than 3.0%.

3.3.3. Sex Determination

Mussel sex was determined by RT-PCR using the sex-specific genes, vitelline envelope receptor for lysine (*Verl*) or vitelline coat lysine (*Vcl*) mRNA expression as described in (Hines *et al.*, 2007, Sedik *et al.*, 2010). Primers are described in Table 3.1.

Table 3.1: PCR conditions for the sex-specific gene method
verl: vitelline envelope receptor for lysine; *vcl*: vitelline coat lysine;
F:Forward;R: Reverse

Gene (Genbank)		Sequence	Annealing	Product (bp)	Reference
Verl (FM995161.1)	F	5'-CTGCAATGGTTTTGGTTGTG-3'	50°C	350	Hines *et al.* (2007)
	R	5'-CCGAAGGAAATGGAACTGAA-3'			
Vcl (FM995162.1)	F	5'-TTGCGTTTCACATGGTTGAT-3'	55°C	250	Sedik *et al.* (2010)
	R	5'-AGAGCTGTTTTGGCCACAGT-3'			

3.3.4. Serotonin level

Serotonin concentrations in S15 fractions were analyzed by ELISA (competitive Serotonin ELISA kit IBL, Germany) according to the manufacturer. Optical density was measured at 405 nm with Spectra Max M5 (Molecular devices). Serotonin concentrations were calculated with a standard curve using a 4-parameter logistics curve (SoftMax Pro Data Acquisition & Analysis, Molecular devices). Detection and quantification limits are 0.014 and 1.50 ng/mL, respectively. Blanks and serotonin controls respected all criteria required by the manufacturer. Low cross-reactivity reaction with 5-hydroxyindoleacetic acid (0.110%), melatonin (0.040%), 5-methoxy-tryptamine (0.015%), and other molecules (<0.01%) could occur. Results are presented as 5-HT ng/mg protein.

3.3.5. Monoamine oxidase (MAO) activity

MAO activity was determined in mantle homogenates diluted in the conservation buffer (1:50) as described by Gagné *et al.* (2007) using tyramine as substrate. The reaction mixture consisted of 100 µM tyramine, 1 µM dichlorofluorescein diacetate, 100 µM aminotriazole and 20 µg/mL horseradish peroxidase in 10 mM HEPES pH 7.4 containing 140 mM of NaCl. The mixture was incubated for 60 min at 30°C and fluorescence emissions were measured at 485/20 nm excitation and 528/20 nm emission every 3 minutes using a spectrofluorimeter (Spectra Max M5, Molecular devices). The enzymatic activity was expressed as RFU/min/mg protein.

3.3.6. Serotonin transporter expression

SERT protein expression was analyzed by Western blot as previously described by Lanoix *et al.* (2012b) (Taylor *et al.*, 2013). Briefly, proteins were extracted from the mantle homogenate with radioimmunoprecipitation buffer (RIPA) containing protease inhibitors (cOmplete ULTRA Tablets, Roche, Laval, Canada) and phosphatase inhibitor cocktail (Thermo Scientific, Burlington, Canada). After sonication, solutions were centrifuged (14,000 x g) at 4°C during 10 min. Protein concentrations were determined with the BCA kit as described by the manufacturer (Pierce Biotechnology, Rockford, CA). Proteins (25 µg) were separated on 10% SDS-PAGE gel and transferred to a polyvinylidene fluoride (PVDF) membrane using Trans-Blot Turbo transfer system (Bio-Rad, Mississauga, Canada). Membranes were blocked with 5% skimmed milk in 0.5% PBS-Tween. Membrane was exposed to anti-SERT (AB-N09, Advanced Targeting Systems, San Diego, USA) primary antibody diluted 1:1000 in 5% PBS skimmed milk for 1.5 h at room temperature (RT), then rinsed 2 times in deionized water, washed 3 times for 5 min in PBS and rinsed 4 times in deionized water. Membranes were then incubated with secondary anti-mouse antibody HRP conjugate (AP192P; Millipore, Etobicoke, Canada) diluted 1:10,000 in 5% PBS skimmed milk for 1h at RT, then rinsed 2 times in deionized water, washed 3 times for 5 min in PBS and rinsed again 4 times in deionized water. Chemiluminescence was detected using Clarity Western ECL Blotting Substrate (Bio-Rad, Mississauga, Canada) and revealed under Chemidoc MP imaging system (Bio-Rad, Mississauga, Canada). Using tools from Image Lab 4.1 software (Bio-Rad, Mississauga, Canada), protein expression and normalization on total proteins were determined. Protein levels were expressed as a ratio of specific band density and total protein stained using Thermo Scientific Pierce Reversible Protein Stain Kit for PVDF membranes (MemCode staining Solution; Thermo Fisher Scientific).

3.3.7. Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Outliers were trimmed with the outlier-labeling rule (Hoaglin *et al.*, 1987, Hoaglin *et al.*, 1986). Distribution normality was tested by skewness (± 2) and kurtosis (± 9) (Hair *et al.*, 2010). Variance homogeneity was verified by Levene's test. ANOVA or Brown-Forsythe (according to equal or unequal observation number, respectively) analyses of variance were used to compare exposure conditions. For non-parametric data, Kruskal-Wallis tests were conducted. Analyses were also made for each sex. Fisher's LDS or multiple Mann-Whitney U was used to make multiple comparisons when a

statistical test was significant. To compare sexes, T-test with normal or non-normal variances was applied. When the data was not normally distributed, Mann-Whitney U test was preferred. SPSS 23.0 was used for all statistical analysis (IBM corp., 2015). $P < 0.05$ was considered statistically significant.

3.4. Results

Artificial seawater, the control vehicle, contained 403 ± 19 nM of Mn and less than the detection limit for Pb and Cd (< 0.32 and < 0.14 nM, respectively; Table 3.2). Metal concentrations expected in each tank after spiking were accurate for the highest concentrations of Mn, Pb, and Cd (Table 3.2). Mn concentrations decreased by 73 ± 8% between water replacements in each exposure tank (Table 3.2), which is also the case, to a lesser extent, for Pb and Cd at 10 nM (9 and 23%, respectively). In used water of 100 and 1000 nM exposure tanks, Pb concentrations were detected (Table 3.2). All mussels were in a post-spawn reproductive cycle stage of which 74% had no gametes in the mantle (data not shown). Globally, 48% of mussels were males and 52% were females (Table 3.3). However, the ratio male/female varies between exposure tanks, ranging between 0.33 and 3.

Table 3.2: Mn, Pb, and Cd concentrations in artificial seawater and experimental tank.
Water was sampled every three days in acid-washed tube after (fresh) and before (used) water replacements. Concentrations were measured by inductively coupled plasma mass spectrometer (ICP/MS).

		Mn concentration (nM)		Pb concentration (nM)		Cd concentration (nM)	
		Fresh	*Used*	*Fresh*	*Used*	*Fresh*	*Used*
Artificial seawater		403 ± 19		< LOD		< LOD	
Control		445 ± 16	136 ± 61	< LOQ	< LOQ	< LOD	0.3 ± 0.2
Mn (nM)	10	376 ± 161	158 ± 71	< LOQ	< LOQ	< LOD	0.3 ± 0.2
	100	542 ± 8	102 ± 31	< LOQ	8 ± 6	< LOD	< LOQ
	1000	1278 ± 8	274 ± 160	< LOQ	6 ± 4	< LOD	< LOQ
Pb (nM)	0.01	444 ± 4	108 ± 52	< LOQ	< LOQ	< LOQ	< LOD
	0.1	522 ± 166	195 ± 62	< LOQ	< LOQ	< LOD	< LOD
	10	435 ± 6	112 ± 76	9.2 ± 0.6	8.4 ± 0.7	< LOD	0.3 ± 0.1
Cd (nM)	0.01	423 ± 12	76 ± 27	1 ± 1	2 ± 1	< LOD	< LOQ
	0.1	430 ± 10	132 ± 129	1.2 ± 0.9	1 ± 1	< LOD	0.5 ± 0.5
	10	442 ± 7	113 ± 75	< LOQ	< LOQ	11 ± 2	8.5 ± 0.3

Average ± standard deviation, n=3-4
< LOD: under detection limit (Mn: 0.58 nM; Pb: 0.32 nM; Cd: 0.14 nM)
< LOQ: under quantification limit (Mn: 1.87 nM; Pb: 0.96 nM; Cd: 0.25 nM)

Table 3.3: Number of male and female mussels in experimental tanks.
Mussel sex was determined by RT-PCR.

		Male	*Female*	*Ratio M/F*
Control		3	9	0.33
Mn (nM)	10	8	4	2
	100	5	7	0.71
	1000	9	3	3
Pb (nM)	0.01	4	8	0.5
	0.1	8	4	2
	10	3	9	0.33
Cd (nM)	0.01	7	5	1.4
	0.1	6	6	1
	10	5	7	0.71
Total		**58**	**62**	**0.94**

For all metals 5-HT levels in the S15 fraction were not significantly different between conditions (Fig. 3.1; Mn: $H = 1.294$, $p = 0.731$; Pb: $H = 3,814$, $p = 0.282$; Cd: $F = 0.670$, $p = 0.575$). No difference was observed between 5-HT levels in male and female mussels exposed to 100 nM Mn ($t = 2.014$, $p = 0.077$). Whereas 5-HT levels in male mussels exposed to 0.01 nM Cd trend to be lower than control, but was not statistically significant (Fig. 3.1C; upper graph; B-FF = 1.398, $p = 0.059$). However, for mussels exposed to 0.01 nM Cd, 5-HT level was significantly lower in males than females (Fig. 3.1C; upper graph; $t=-2.734$; $p=0.029$). Pb exposure did not affect 5-HT levels when sexes were separated (Fig. 3.1B).

MAO activity was two times higher in females than males in blue mussel mantle (Fig. 3.2; upper graphs; $t=-4.488$, $p=0.001$). MAO activity was not significantly affected by Mn or Pb (Fig. 3.2A and 2B; Mn: $F = 0.784$, $p = 0.510$; Pb: $F = 0.175$, $p = 0.913$), whereas it was decreased by 0.1 nM Cd in mussels mantle in both sexes (Fig. 3.2c; $F = 9.468$, $p < 0.0001$). Sex-separated results showed that MAO activity. Considering each sex separately, Mn exposure did not significantly change MAO activity (Fig. 3.2A; upper graph; male: B-FF=2.147, $p=0.127$; female: B-FF=1.893). However, at 0.1 nM Pb, MAO activity was significantly lower than control for females (B-FF = 3.819, $p = 0.027$) and higher than control for males (B-FF = 4.183, $p = 0.031$) (Fig. 3.2B; upper graph). For blue mussels exposed to Cd (Fig. 3.2C; upper graph), a non-monotonic dose response was observed for males with an increase MAO activity at 0.01 and 10 nM Cd (B-FF = 16.706, $p <0.0001$), whereas for females, a decrease was observed at 0.1 nM Cd (B-FF = 4.609, $p = 0.018$).

All concentrations of Mn, Pb, and Cd exposure significantly decreased SERT protein expression in blue mussel mantles (Fig. 3.3A-C; Mn: $H = 10.248$, $p = 0.017$; Pb: $H = 9,257$, $p = 0.026$; Cd: $F = 5.323$, $p = 0.008$), excepted at 10 nM Mn. Even though this trend was repeated when sexes were separated (upper graphs), only females exposed to Mn had statistically significant lower SERT protein expressions (Fig. 3.3A; upper graph; Mn female: $H = 8.018$, $p = 0.046$).

3.5. Discussion

This study demonstrates that low environmental concentrations of Mn, Pb and Cd for 28 days can disrupt the 5-HT system in blue mussel *Mytilus edulis.* The decrease of SERT protein expression observed in the mantle of the *Mytilus edulis* exposed to metals, when compared to control, could limit the transport of 5-HT in cells altering mussel physiology. SERT protein expression is regulated by intracellular concentrations of calcium $[Ca^{2+}]_i$ (Jayanthi *et al.*, 1994,

Seimandi *et al.*, 2013), and Pb, Mn, and Cd are known to interact with Ca (ATSDR, 2004b, Lafond *et al.*, 2004, Tchounwou *et al.*, 2012). Thus, metals could alter SERT protein expression by perturbing the $[Ca^{2+}]_i$. Metals might also interact with diverse protein functional groups such as sulfhydryl and amine, which could affect SERT function (ATSDR, 2004b, Disbudak *et al.*, 2002, Zawia *et al.*, 2000) and limits the transport of 5-HT in cells, altering mussel physiology. Further studies are needed to investigate the mechanism involved in the effect of metal on SERT expression and if they affect SERT activity.

Long-term exposure to low concentrations of Pb, Mn and Cd decreased SERT expression, but did not affect 5-HT levels in the mantle of the blue mussel. This is in accord with other studies showing that 5-HT levels is decreased after an acute 24 h exposure to Pb and Cd, but return to control levels after 120 h exposure in *Perna perna* muscle (Almeida *et al.*, 2003). Decrease in 5-HT levels in *Anadonta cygnea* exposed for 14 h to 1000 nM of Pb or Cd also a recovery over time (Salanki *et al.*, 1990). These and our studies suggest that cells could return rapidly to 5-HT homoeostasis using less effective transports such as organic cation transporters which are able to transport 5-HT (Courousse and Gautron, 2015; Hansson et al., 2009). Further investigations are needed to verify this hypothesis. A perturbation of available L-tryptophan for 5-HT synthesis could also explain the unchanged 5-HT levels. L-tryptophan is involved in the kynurenine pathway through the indoleamine 2,3-dioxygenase (IDO), which the activity is increased by prostaglandins synthesized by cyclooxygenase (COX; also known as prostaglandin-endoperoxide synthase (PTGS)) (Muller *et al.*, 2007). In our study, this pathway seems not involved, since COX activity was not affected by Mn, Pb, and Cd exposure (Supplementary data; Fig. 3.S1).

MAO activity in blue mussel mantle is higher in females than males. This is also observed in humans, where women have higher MAO activity than men (Murphy *et al.*, 1976, Robinson *et al.*, 1971, Sandler *et al.*, 1981). MAO activity in mantle mussels is affected differently by exposure to Cd and Pb between sexes, e.g. it is increased for males and decreased for females in mussels exposed to 0.1 nM Pb. This shows that analyzing mussel MAO activity without distinguishing the sexes alters data interpretation, since results can be the opposite from one sex to the other. Sex can also influence metal accumulation (Aarab *et al.*, 2006, Cubero-Leon *et al.*, 2010, Dang *et al.*, 2012, Mergler, 2012). Richir *et al.* (2014) showed that some trace metals concentrations in *Mytilus galloprovincialis* differ significantly between sexes, such as for Mn and Cd, but not for Pb. Thus, sex identification should always be included in metal exposure studies.

Pb affected MAO activity (female), in a biphasic U-shape way. Cd exposure also showed non-monotonic effect in MAO activity (differing between sexes). Pb and Cd are well known for their endocrine (estrogenic-like) effects (Dyer, 2007, Ricciardi *et al.*, 2008, Stoica *et al.*, 2000). Moreover, Pb decreases the expression of the vitellogenine-like protein in mussel mantle (Supplementary data; Fig. 3.S2), showing an endocrine activity (Gonzalez-Rey *et al.*, 2014b). Low concentrations of endocrine-disrupting chemicals often disturb biological endpoints in non-monotonic dose-response way (reviewed by Vandenberg *et al.* (2012)). Vandenberg *et al.* (2012) did a review on possible mechanisms that can explain why endocrine-disrupting chemicals produce non-monotonic dose-response curves, which include receptor selectivity, competition and down-regulation.

The commercial salt water (Instant Ocean®), used as control (vehicle), already contained low levels of Mn and Pb, demonstrating the importance of characterizing metal concentrations in the control/vehicle water. Nonetheless, since control mussels were exposed to the background metal concentrations, our results demonstrated the effects of metal concentration added. Analyzing metal concentrations in mussel tissues before and after the experiment would have confirmed that metals reached the mantle and demonstrated which proportion of metal was assimilated. Nevertheless, the effect observed on the biomarkers of exposure, lipid peroxidation (LPO), COX and vitellogenin-like protein, demonstrated that the low Mn, Pb, and Cd concentrations used to enter the mussels and was bioavailable (Supplementary data Fig. 3.S1-S3) (Coassin *et al.*, 1992, Gagné *et al.*, 2010b, Olszowski *et al.*, 2015). Moreover, Mn concentrations decreased within each water renewal. Mn ions are unlikely to adsorb on polyethylene surface (Shendrikar *et al.*, 1976). This could suggest that mussels bioaccumulate Mn, which could be associated with the importance of this essential metal in different biological systems (Goldhaber, 2003).

The effects observed could reflect an exposure to a mixture of several metals. In 100 and 1000 nM Mn exposure tanks, Pb is detected in used water. In a previous study, low Mn concentrations in mussels fished in industrial sites with high Pb concentrations were observed (Fraser *et al.*, 2011). In rabbit, a mix exposure of Mn and Pb increased the half-life of Pb in blood (ATSDR, 2004b, Momoko *et al.*, 1984). These suggest that Mn and Pb could interact and compete for same sites in mussels, such as demonstrated in rat brains (Kalia *et al.*, 1984). Thus, Mn could dislodge Pb accumulated in mussels. However, further study analyzing the simultaneous bioaccumulation of these metals in *Mytilus edulis* should be done to confirm this

hypothesis. Study limitations remind us that environmental exposure to a metal is unlikely to happen without being accompanied by exposure to other metals.

3.6. Conclusion

Low environmental concentration of metal disrupts the 5-HT system in blue mussel *Mytilus edulis*. Since serotonin system regulates mussel sexual differentiation, gamete production and spawning (Aiello, 1965; Fong et al., 2003; Gibbons and Castagna, 1984; Gies, 1986; Ram et al., 1999), our results suggest that Mn, Pb and Cd can alter blue mussel reproduction process. This study also emphasizes the importance of sex effect in metal exposure and ecotoxicology studies.

3.7. Acknowledgments

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3.8. Figures

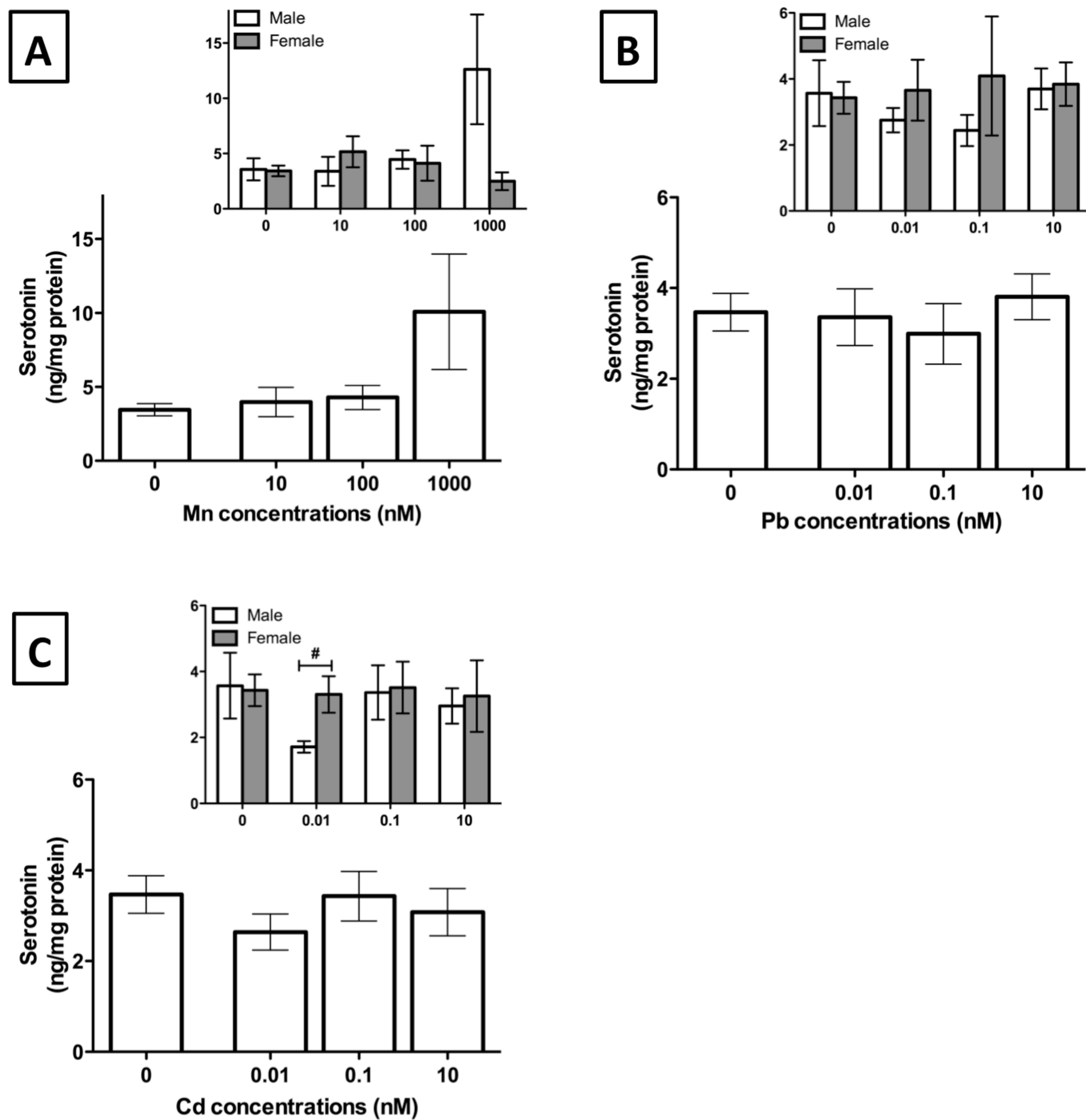

Figure 3.1: Serotonin (5-HT) levels in the mantle of mussels exposed to increasing concentrations of Mn (A), Pb (B), and Cd (C) for 28 days.

Upper graphs show results by sex. No significant differences were observed between exposure tanks (Kruskal-Wallis or Brown-Forsythe, P>0.05). # indicates a significant difference between sexes (T-Test, P <0.05). Results are expressed as the means ± SEM. 0 represents the control.

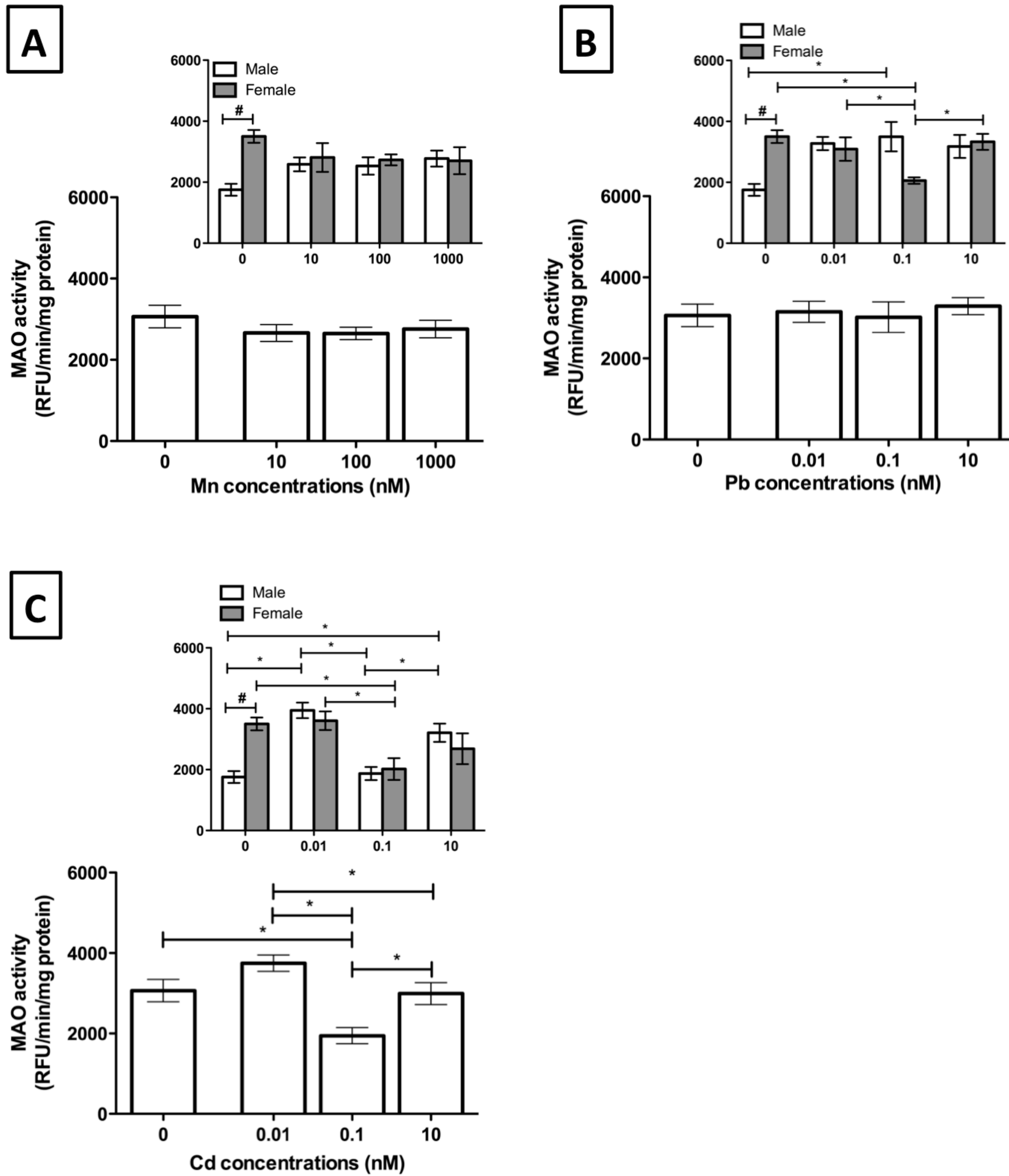

Figure 3.2: MAO activity in the mantle of mussels exposed to increasing concentrations of Mn (A), Pb (B), and Cd (C) for 28 days.

Upper graphs show results by sex. No significant differences were observed between exposure tanks for Mn and Pb (ANOVA, P >0.05). Mussels exposed to 0.1 nM Cd had lower MAO activity. MAO activity was higher in female mussels compared to male in the control tank. * represents a significant difference between exposure (ANOVA or Brown-Forsythe, P <0.05); # indicates a significant difference between sexes (T-Test, P <0.05). Results are expressed as the means ± SEM. 0 represents the control.

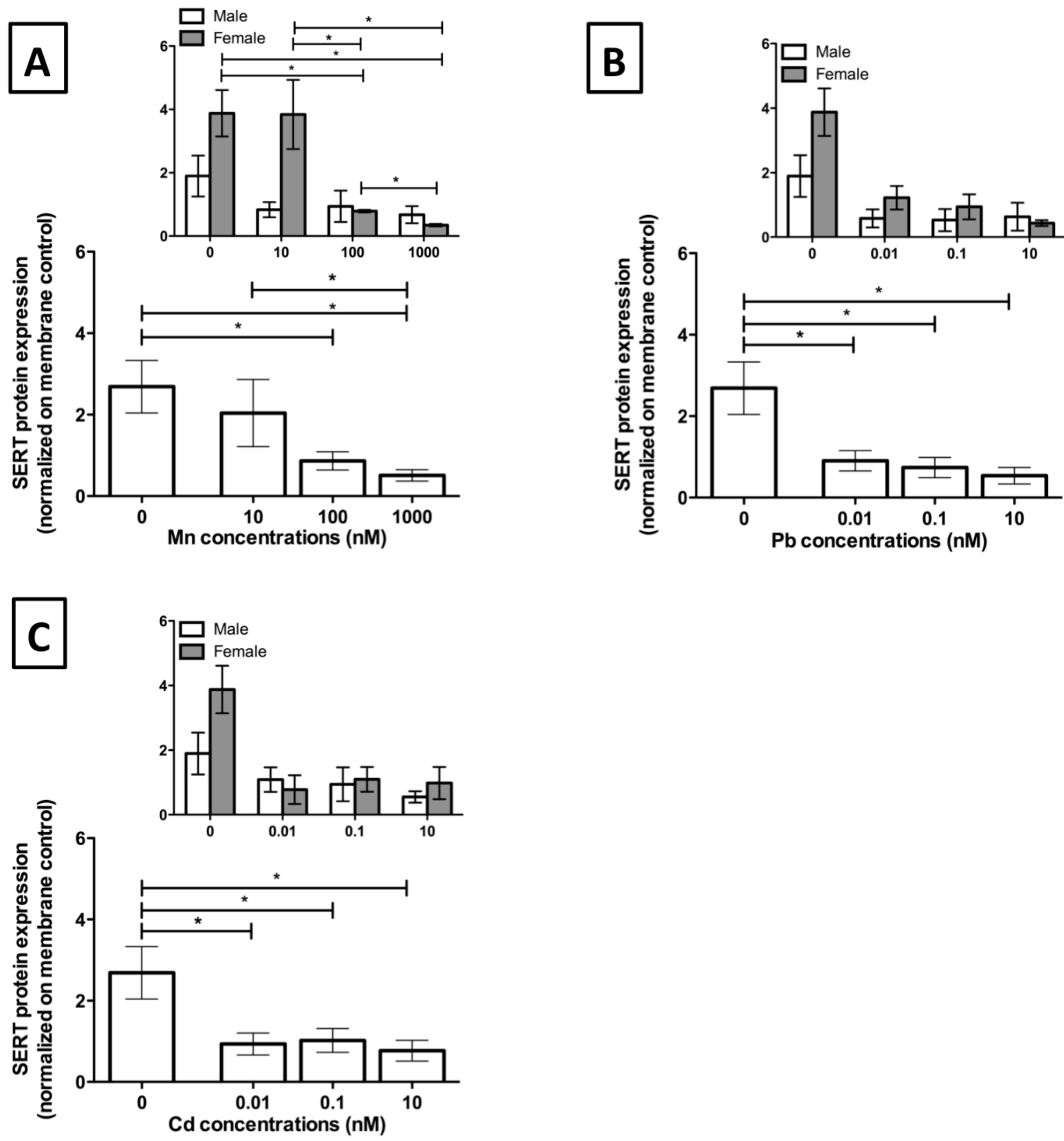

Figure 3.3: SERT protein expression in the mussel mantle decreased after an exposure to increasing concentrations of Mn (A), Pb (B), and Cd (C) for 28 days.

Upper graphs show results by sex. * represents a significant difference between exposure (ANOVA, Kruskal-Wallis or Brown-Forsythe, P <0.05). No significant differences were observed between sexes (T-Test, P>0.05). Results are expressed as the means ± SEM. 0 represents the control.

3.9. Supplementary data

Table 3.S1: Survival rate of mussel during acclimatization and experiment period

		Acclimatization period			Experimental period		
		Dead mussel	***Survival %***	***N***	***Dead mussel***	***Survival %***	***N***
Control		0	100	14	0	100	14
Mn (nM)	10	0	100	14	0	100	14
	100	0	100	14	1	92.9	14
	1000	0	100	14	0	100	14
Pb (nM)	0.01	0	100	14	1	92.9	14
	0.1	1	92.9	14	1	92.3	13
	10	0	100	14	1	92.9	14
Cd (nM)	0.01	0	100	14	1	92.9	14
	0.1	0	100	14	0	100	14
	10	0	100	14	0	100	14
Total		1	99.3	140	5	96.4	139

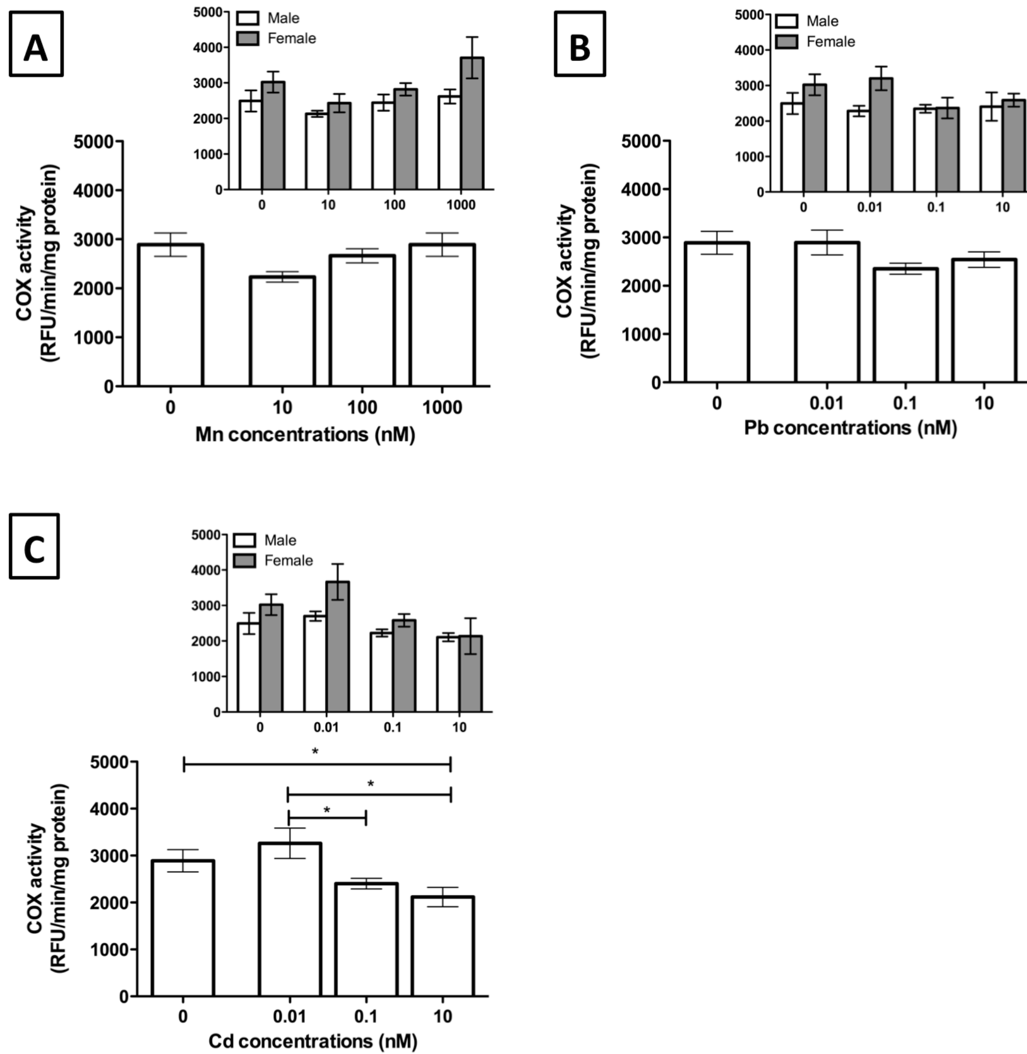

Figure 3.S1: Cyclooxygenase (COX) activity in the mantle of mussels exposed to increasing concentrations of Mn (A), Pb (B), and Cd (C) for 28 days.

COX activity, an inflammation indicator, was analysed as described by Gagné *et al.* (2011b) and Fujimoto *et al.* (2002). COX activity is not significantly altered by Mn (F = 2.666, p = 0.059) neither by Pb (F = 0.178, p = 0.165), whereas it is significantly decreased by 10 nM of Cd (F=4.784, p=0.006). Mussel sex (upper graphs) did not affect COX activity in mantle. * represent a significant difference between exposure tanks (ANOVA or Brown-Forsythe, P <0.05). Control (without metal treatment) is represented by 0 nM.

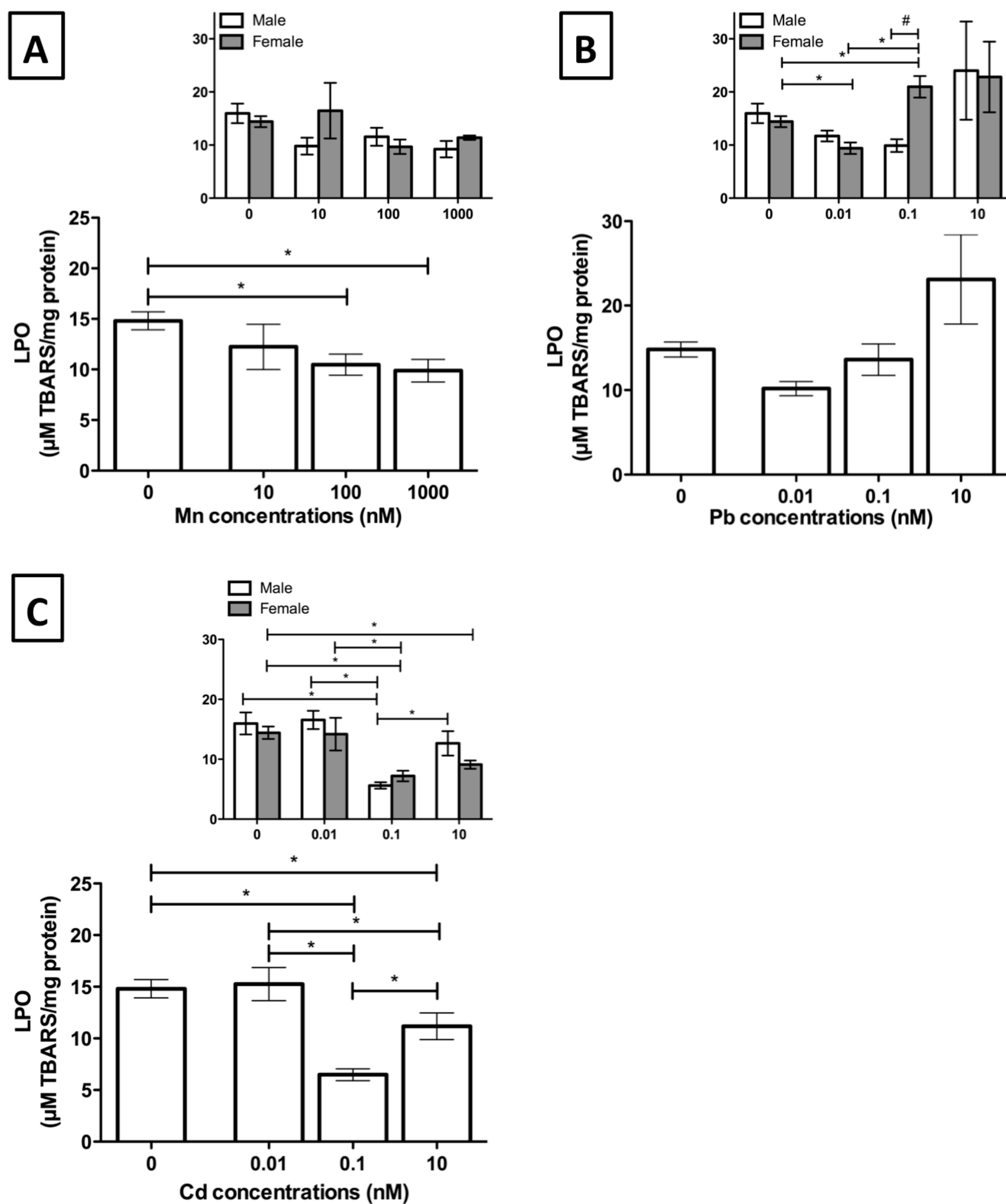

Figure 3.S2: Lipid peroxidation (LPO) levels in the mantle of mussels exposed to increasing concentrations of Mn (A), Pb (B), and Cd (C) for 28 days.

LPO, a biomarker of oxidative stress, was determined by the thiobarbituric acid method (Wills, 1987). LPO is significantly decreased compared to control in mussels exposed to 100 and 1000 nM of Mn (H = 8.773, p = 0.032) and to 0.1 nM and 10 nM of Cd (H = 23.625, p < 0.0001). LPO is not significantly affected by Pb (H = 7.772, p = 0.051) when both sex are considered, but is significantly increased in female mussels by 0.1 nM Pb (H = 11.329, p = 0.010). LPO levels are significantly different between male and female mussels exposed at 0.1 nM Pb (t = -5.021, p = 0.001). Cd decreased LPO levels at 0.1 nM for males and females (male: B-FF = 9.530, p = 0.002; female: H = 12.683, p = 0.005). * represent a significant difference between exposure tanks (Kruskall-Wallis or Brown-Forsythe, P <0.05), while # indicates a significant difference between sexes (T-Test, P <0.05). Results are expressed as the means ± SEM. Control (without metal treatment) is represented by 0 nM.

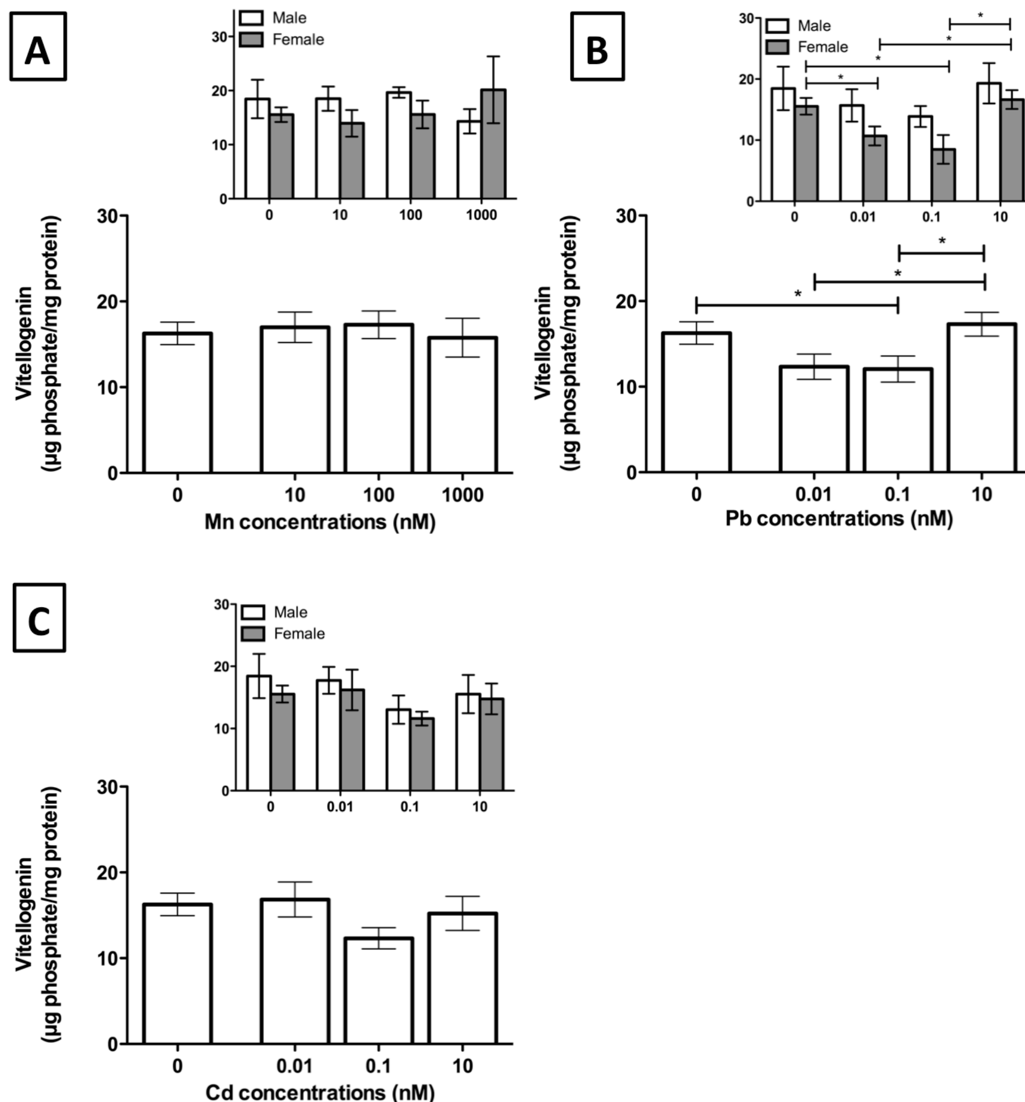

Figure 3.S3: Vitellogenin-like protein levels in the mantle of mussels exposed to increasing concentrations of Mn (A), Pb (B), and Cd (C) for 28 days.

Vitellogenin-like protein, an endocrine effect bioindicator, levels was determined by the alkali-labile phosphate (ALP) assay as described by (Gagné *et al.*, 2010a). (B) Vitellogenin-like protein levels is decreased in male and female mussels at 0.1 nM Pb (F=3.539, p=0.022), but was only significant in female mussels for the 0.01 and 0.1 nM Pb concentration (B-FF=4.856, p=0.012). (A-C) Mn and Cd did not affect significantly vitellogenin-like protein levels even when sexes were taken separately. * represent a significant difference between exposure tanks (ANOVA or Brown-Forsythe, P <0.05). Results are expressed as means ± SEM. Control (without metal treatment) is represented by 0 nM.

CHAPITRE 4 : MANGANESE AND LEAD DISTURB THE SEROTONIN SYSTEM OF HUMAN PLACENTA

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Contribution de l'étudiant

L'étudiant a participé à l'élaboration de l'étude, réalisé toutes les expériences et analysé les résultats dans cet article. Il a également rédigé l'article et participé au choix du journal de publication.

Résumé de l’article en français

Une exposition maternelle aux métaux peut interférer avec le fonctionnement du placenta, traverser la barrière placentaire et ainsi altérer, directement et indirectement, le développement du fœtus et de l’embryon. Les métaux lourds, tels que le plomb Pb, et les métaux essentiels comme le Mn peuvent altérer le système 5-HT. Le placenta possède un système sérotoninergique qui est essentiel au bon déroulement de la grossesse, aux fonctions placentaires ainsi qu’au développement fœtal. Ainsi, l’objectif de cette étude est d’évaluer l’effet de faibles concentrations de Pb et de Mn sur le système 5-HT de cellules BeWo (*in vitro*) et de tissus placentaires humains. Des cellules de choriocarcinome (lignée BeWo) ont été exposées à des concentrations croissantes de Mn et de Pb (10 à 2000 nM et 0,01 à 1000 nM, respectivement) avant que le système 5-HT n’ait été caractérisé. L’expression du transporteur de la sérotonine (SERT) et de son récepteur 2A ($5\text{-}HT_{2A}$) a été évaluée dans les placentas de 31 femmes après l’accouchement par rapport aux concentrations de Pb et Mn dans le placenta, le sang maternel et le sang du cordon. Les résultats ont montré que l’activité spécifique de SERT dans les cellules BeWo exposées aux deux métaux a été diminuée, ce qui a été associé à une diminution des concentrations de 5-HT intracellulaire. L’expression de la protéine SERT a été diminué dans les cellules BeWo exposées au Pb et était plus faible dans les placentas contenant plus de Pb dans le sang de cordon; l’expression de l’ARNm codant le récepteur $5\text{-}HT_{2A}$/*HTR2A* était plus basse dans les placentas contenant plus de Pb. Aucune concentration de Mn dans le sang (maternel ou du cordon) et dans le placenta n’a interféré avec l’expression de SERT et 5-HT2AR. Ces résultats suggèrent que le Mn et le Pb peuvent affecter le système 5-HT placentaire, ce qui pourrait influer la santé de la grossesse et le développement du fœtus.

MANGANESE AND LEAD DISTURB THE SEROTONIN SYSTEM OF HUMAN PLACENTA

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4.1. Abstract

Maternal environmental exposure to metals may interfere with placental functions, cross the placental barrier and thus disturb, directly and indirectly, embryo and fetal development and programming. Heavy metals, such as lead (Pb), and essential metals, like manganese (Mn), can disturb the serotonin (5-HT) system. The placental 5-HT system is essential for healthy pregnancy, placental function and fetal development. The objectives of this study were to examine the placental 5-HT system with respect to Pb and Mn in BeWo cells (*in vitro*), and in human placental tissue. Choriocarcinoma BeWo cells were exposed to increasing Mn and Pb concentrations (10 to 2000 nM and 0.01 to 1000 nM, respectively) prior to 5-HT system characterization. Serotonin transporter (SERT) and 5-HT receptor 2A (5-HT_{2A}R) expressions at delivery were assessed in the placentas of 31 women with respect to Pb and Mn concentrations in the placenta, maternal blood and cord blood. Results showed that specific SERT activity is decreased in BeWo cells exposed to both metals, which was associated with lower 5-HT levels in cells. SERT protein expression was decreased in BeWo cells exposed to Pb and was lower in placentas with cord blood containing higher Pb; *5-HT_{2A}R/HTR2A* mRNA expression was lower in the placenta containing higher Pb. No Mn concentration in blood (maternal and cord) and placenta interfered with SERT and 5-HT2AR. These findings suggest that Pb and Mn may affect the placental 5-HT system, which could influence pregnancy health and fetal development.

Highlights

- SERT activity and serotonin levels are decreased in BeWo cells exposed to low concentrations of Pb and Mn.
- SERT protein expression is decreased in BeWo cells exposed to Pb and is lower in placentas in contact with cord blood containing more Pb.
- 5HT2AR mRNA expression is lower in placental tissues with more Mn.

Keywords

SERT, 5-HT$_{2A}$R, BeWo, MAO, COX, maternal blood, cord blood, trophoblast

4.2. Introduction

It is well known that metal exposure can lead to serious health conditions (Jarup, 2003). Lead (Pb), a heavy metal that can be found, *inter alia*, in tobacco smoke (Richter *et al.*, 2013), is associated with neurological, hematological, and cardiovascular effects (ATSDR, 2004b). At high concentrations, manganese (Mn), an essential metal implicated in enzyme functions, can induce Parkinson-like symptoms called manganism (Grandjean *et al.*, 2006, Mergler, 1999). Both metals are associated with central nervous system perturbations which could lead to neurologic and neuropsychiatric disorders (Bouchard *et al.*, 2011, Grandjean *et al.*, 2006, Neal *et al.*, 2013). Mn and Pb can alter the serotonin system, a neurotransmitter, in animal brains (Bonilla *et al.*, 1984, Nation *et al.*, 1989, Widmer *et al.*, 1991), and in humans (Ogawa *et al.*, 2006).

Serotonin (5-hydroxytriptamine, 5-HT) acts also as a growth factor and a hormone. In humans, 5-HT is well known for its role in mental health and psychiatric diseases such as depression and attention deficit hyperactivity disorder (Hoyer *et al.*, 2002). 5-HT also participates to immune and inflammatory responses (Shajib *et al.*, 2015). This monoamine is synthesized from L-tryptophan by a series of reactions rate-limited by tryptophan hydroxylase enzymes (TPH) (Vrana *et al.*, 1993). There are seven 5-HT receptor classes including 5-HT$_{2A}$R, which is involved in the placental development (Arseneault *et al.*, 2005, Oufkir *et al.*, 2010). 5-HT transport in cells occurs mostly by its spectific transporter (SERT) (Blakely *et al.*, 1991). Monoamine oxidases (MAO) degrade 5-HT into 5-hydroxyindoleacetic acid (5-HIAA) (Jonnakuty *et al.*, 2008).

The 5-HT system is expressed in the placenta and contributes to its development (Bonnin *et al.*, 2011a, Oufkir *et al.*, 2010, Viau *et al.*, 2009). It has been suggested that maternal and placental 5-HT, that may transit through placental SERT, is essential for early fetal heart and brain development (Hoyer *et al.*, 2002, Nation *et al.*, 1989, Ogawa *et al.*, 2006). Thus, a 5-HT system disruption in the placenta could lead to problems during pregnancy and possible adverse fetal health outcomes (reviewed in St-Pierre *et al.* (2015)).

Important physiological changes, as well as increased nutriment demands during pregnancy, mobilize metals in the maternal blood, which could lead to elevated prenatal Mn and Pb prenatal (Gulson *et al.*, 1997, Takser *et al.*, 2004b). It has been shown that *in utero* exposure to Pb or Mn can disturb fetal growth and cognitive development (Hu *et al.*, 2006, Ostrea *et al.*, 2002, Takser *et al.*, 2003). Metals can act directly on the fetus by crossing the placenta or they can alter placental functions (Hu *et al.*, 2006, Lafond *et al.*, 2004). The placenta is essential for pregnancy and fetal development by its immune, metabolic and endocrine functions (Alsat *et al.*, 1999, Bauer *et al.*, 1998).

Only two studies demonstrated an association between metals and human placental 5-HT system, both focussing on Mn. Takser *et al.* (2003) observed negative correlation between maternal blood Mn and 5-HIAA levels in cord blood. Abdelouahab *et al.* (2010) showed positive correlations between MAO activity in the placenta and maternal and cord blood Mn concentrations. The aim of the present study is to evaluate the effects of Pb and Mn on the 5-HT system of the human placenta, using an *in vitro* model, a human trophoblast choriocarcinoma cell lines isolated from a boy fetus (BeWo) and a sub-sample of a birth cohort study of healthy pregnant women.

4.3. Materials and methods

4.3.1. *In vitro* model

BeWo cells (ATTC, Manassas, VA) were cultured in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum (DMEM/F12, Sigma-Aldrich, ON, Canada). Cells were kept in 75 cm^2 flasks at 37°C in an atmosphere of 5% CO_2. All experiments were done on cells aged between 8 and 20 passages. 300,000 cells were transferred in 6-well plates for metal exposure, except for SERT activity (75,000 cells in 24-well plate). Each experiment was done at least 3 times.

$PbCl_2$ and $MnCl_2$ (Sigma-Aldrich, ON, Canada) solutions were freshly prepared for each experiment or replicate in culture medium or the SERT activity buffer. Metal concentrations were chosen to reflect levels found in unexposed women of other previous studies (Pb: 0.01 to 1000 nM; Mn: 10 to 2000 nM) (Abdelouahab *et al.*, 2010, Esteban-Vasallo *et al.*, 2012, Osman *et al.*, 2000). Time course were made to establish metal exposure time for each experiment (data not shown).

4.3.2. Placental tissues

The original study of pregnant women has been previously decribed (Morrissette *et al.*, 2004, Takser *et al.*, 2004a, Takser *et al.*, 2004b). Briefly, pregnant women were recruited at prenatal clinics in the southwestern region of Québec, Canada, between 2000 and 2002. All women provided their written informed consent. Questionnaires were administered to obtain information on medical history, work, smoking and drinking habits during pregnancy as well as physical features (age of mother, gestational age and weight gain). Newborn sex was also noted. The placental index was calculated with the weight of the newborn and the placenta (newborn weight / placenta weight).

A trained nurse collected blood samples in heparinized tubes at the first and second trimester. At delivery, maternal and umbilical cord blood were also collected. Plasma was separated by centrifugation and transferred to glass tubes. All plasma samples were frozen at -20°C until analysis. Amniotic and chorionic membranes were removed from the placenta and 5 cm^2 pieces were randomly taken, frozen in liquid nitrogen and stored at -80°C until analysis. Metal concentrations were determined at the Centre de Toxicologie du Québec (QC, Canada) by inductively coupled plasma-mass spectrometry, for placenta tissues, and by graphite furnace atomic absorption spectrometry for maternal and umbilical cord blood samples.

Exclusion criteria for the present study included occupational exposure to toxic substances, medication, pathologies, twins, and multi-fetal gestational outcomes. The Ethics Committee of the University of Québec in Montréal approved.

4.3.3. RT-qPCR

For *in vitro* study, total RNA was extracted from BeWo cells exposed 24h to Mn or Pb with the AllPrep RNA kit (Qiagen, ON, Canada) according to the manufacturer’s protocol. Total RNA was extracted from 100 mg of frozen placental tissues using the Rneasy Mini kit (Qiagen, ON,

Canada) with the manufacturer's instructions and kept at -80°C. Primers used are described in table 4.1. All details are provied in Supplementary Material.

Table 4.1: Specific gene primer sequences for qPCR

Gene	GenBank number	Sequences		Product (bp)
		Forward (5' to 3')	Reverse (5' to 3')	
HTR2A/ 5-HT$_{2A}$R	NM_000621	AGCTTCCTCCCTCAGAGTTCTT	GGGCACCACATCACCACAAA	170
SLC6A4 SERT/	NM_001045	CCGCCACAACTACGACTT	CTGTTGGTGTTTCTGGGGTAAT	178
TBP	NM_003194	CACGAACCACGGCACTGAT	TTGGGTGGGTGAGCACAAGG	215
HPRT1	NM_000194	GACCAGTCAACAGGGGACATAA	AAGCTTGCGACCTTGACC	167
TOP1	NM_003286	GGCGAGTGAATCTAAGG	CTTAAAGGGTACAGCGAATG	90

4.3.4. Western blot

For the *in vitro* model, BeWo cells were exposed 24 h to metals before protein extraction. For the tissue analysis, proteins were extracted from frozen placental tissues. Extraction protocols are presented in Supplementary Material.

Protein concentrations were determined with the Pierce™ BCA Protein Assay Kit using the manufacture's instruction (Pierce Biotechnology, Rockford, CA). 40 µg (placental tissues) or 20 µg (*in vitro*) of proteins were separated on 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were then blocked with 5% skimmed milk in 0.5% TBS-Tween before incubations with antibodies (details described in Supplementary Material).

For *in vitro,* protein expression and normalization on total proteins, using Reversible Protein Stain Kit for PVDF membranes (Pierce Biotechnology, Rockford, CA) were determined with the Image Lab 4.1 software (Bio-Rad, Mississauga, Canada).

For tissues analysis, protein expressions are shown as the density ratio of the interest protein specific band on the density of GAPDH reference protein band.

4.3.5. SERT activity

SERT activity was measured in BeWo cells following a protocol adapted from Qian *et al.* (1997). Cells were plated at 75,000 cells/well in 24 well culture plates with normal culture medium one day before the experiment to allow adherence. More details are provided in Supplementary Material.

4.3.6. Serotonin Levels

BeWo were exposed 2 h to $MnCl_2$ or $PbCl_2$. The culture media were placed in opaque plastic tubes and centrifuged 5 min at 14,000 g to remove impurities. Supernatants were stored at -80°C until further analysis. Cells were washed three times with cold PBS on ice before being detached in PBS (150 µL) and stored at -80°C. Protein concentrations were determined as described before.

Serotonin concentrations in medium and cells were measured with a Serotonin ELISA kit (IBL, Germany) according to the manufacturer's instructions. Optical density was measured at 405 nm with Spectra Max M5 (Molecular devices). Serotonin concentrations were calculated with a standard curve using a 4-parameter logistics curve. Detection and quantification limits are 0,014 and 1,50 ng/mL, respectively. Blanks and serotonin controls respected all criteria required by the manufacturer. Low cross-reactivity reaction with 5-Hydroxyindoleacetic acid (0,110%), melatonin (0,040%), 5-methoxy-tryptamine (0,015%), and other molecules (<0,01%) could occur. Results were normalized on protein concentrations and on control.

4.3.7. Monoamine Oxidase

BeWo were exposed as in serotonin levels section. However, after washing, cells were detached in 350 µL before the determination of protein concentrations. Monoamine oxidase (MAO) activity was determined in cell solution as described in Gagné *et al.* (2007) using tyramine as substrate with a spectrofluorimeter (Spectra Max M5, Molecular devices) (Details in Supplementary Material). The enzymatic activity was expressed as the increase in relative fluorescence units (RFU)/min/mg proteins before normalization on control.

4.3.8. Statistical analysis

All statistical analyses were conducted with JMP 9.0 (SAS Institute Inc., 2010).

For the *in vitro* model, results are presented as mean ± SEM. Non-parametric Kruskal-Wallis tests were conducted to compare all metal concentrations. Multiple Wilcoxon tests were used to make multiple comparisons. When a tendency was suspected in results, Wilcoxon tests were applied to compare specific concentrations with control and a Benjamini-Hochberg correction was applied. $P < 0.05$ was considered statistically significant.

For the placental tissue analysis, linear regressions and Wilcoxon tests were used to find relations between gene or protein expressions and each of the potential covariable (maternal age, maternal weight gain, gestational age at birth, fetal sex, placental index, tobacco consumption during pregnancy and TSH levels in the second trimester). Then, SERT and 5-HT_{2A}R expressions (proteins and genes) were adjusted for significant covariates using multiple regression models. The median of Pb and Mn concentrations in each biological compartment were used as a threshold to separate two groups of exposure in order to evaluate the possible effects of those metals. Wilcoxon tests were applied to compare group with more metal to group with less metal. Missing data were not included in statistical test. Results are presented as adjusted mean ± standard error of the mean (SEM). $P < 0.05$ was considered statistically significant. $0.1 > P > 0.05$ was used to show tendencies.

4.4. Results

4.4.1. *In vitro* study

Metals concentrations used for BeWo exposure were not cytotoxic (Supplementary data; Fig. 4.S1) and reached the cells (Supplementary data; Fig. 4.S2). Metal exposures did not change BeWo *5-HT_{2A}R/HTR2A* mRNA expression (Fig. 4.1A and 1B, Mn: $H=2.688$, $p=0.748$; Pb: $H=2.892$, $p=0.822$), and 5-HT_{2A}R protein expression (Fig. 4.1C and 1D, Mn: $H=1.561$, $p=0.906$; Pb: $H=2.113$, $p=0.909$). *SERT/SLC6A4* mRNA expression was not altered by Mn and Pb concentrations (Fig. 4.2A and 2B; Mn:$H=2.591$, $p=0.763$; Pb: $H=2.580$, $p=0.859$). SERT protein expression decreased in BeWo exposed to 0.1 nM Pb when compared to control (Fig. 4.2D; $W=4.0833$, $p=0.0433$). A significant decrease of 25 to 50% was observed in BeWo SERT activity after 2h exposure to 1000 nM and 2000 nM Mn (Fig. 4.2E; $H=24.991$, $p < 0.0001$) and with all Pb concentrations (Fig. 4.2F; $H=4.673$, $p=0.023$).

5-HT levels were decreased in BeWo cells after the Mn or Pb exposure (Fig. 4.3A and 3B; Mn: W=4.500, p=0.0339; Pb: W=3.8571, p=0.0495), but not in medium (Fig. 4.3A, Mn: H=3.480, p=0.626; Pb: H=1.991, p=0.920). MAO activity in BeWo was not affected by Mn exposure (Fig. 4.3C; H=10.940, p=0.053), but was decreased after an exposure to 1000 nM Pb (Fig. 4.3D; W=8.0667, p=0.0045).

4.4.2. Placental tissue analysis

The main population characteristics are presented in table 2. Thirty-one woman, aged between 17 and 35 years old (average 25 ± 5 yr), were included in the study. Nine women reported smoking during pregnancy. Sex distribution of babies was almost equal (15 boys and 13 girls ; 3 was missing). Women did not have metal exposure history (work, activities, etc.; data not shown). Other cofactors that could influence results are present in tables 2 and S1 (Supplementary Material).

Table 4.2: Population characteristics

	n	Ratio or Mean ± SD (range)
Total number of participants	31	
Maternal age (years)	28	25 ± 5 (17 – 35)
Maternal weight (lb)	27	181 ± 25 (133 – 215)
Maternal weight gain (lb)	27	35 ± 15 (5 – 68)
Tobacco used during pregnancy (Yes/No)	28	9/19
Gestational age at birth (weeks)	28	39 ± 2 (36 – 42)
Placental index (newborn weight / placenta weight)	27	6 ± 1 (4.2 – 8.0)
Fetal sex	28	15 boys / 13 girls

Mn and Pb concentrations in maternal blood, placenta and cord blood were all significantly different from each other (Table 4.3; Mn: H=60.41, p <0.0001; Pb: H=39.09, p <0.0001). Metal concentrations were closed to concentrations found in other studies of unexposed pregnant and non-pregnant women (Abdelouahab *et al.*, 2010, Esteban-Vasallo *et al.*, 2012, Kim *et al.*, 2015, Osman *et al.*, 2000, Thomas *et al.*, 2015). Mn concentrations were higher in the placenta (1247 ± 422 nM) compared to blood concentrations (Table 4.3). The opposite was observed for Pb, where placental Pb concentrations (26 ± 29 nM) were lower than those in blood (Table 4.3). Correlations between metals are shown in Supplementary Material (Table 4.S2).

Table 4.3: Mn and Pb concentrations (nM) in maternal blood, cord blood, and placenta at delivery.

	Mn (nM)			**Pb (nM)**		
	Maternal	Placenta	Cord	Maternal	Placenta	Cord
n	26	25	31	26	31	26
Mean	**320**	**1247**	**692**	**89**	**26**	**60**
SD	107	422	296	36	29	33
Median	**305**	**1301**	**613**	**100**	**20**	**50**
Distribution	182-675	557-2787	371-1629	<50-150	<2.5-49	<50-150
Detection limit	2	2	2	50	2.5	50

SERT protein expression was lower in placentas with more Pb in cord blood (W=6.2891, p=0.0121), while the difference with Pb in placenta showed a tendency, but did not reach the significance threshold (Fig. 4.4D, W=3.5162, p=0.0608). Placental *5-HT$_{2A}$R/HTR2A* mRNA expressions tended to be lower in maternal blood with more Mn and Pb (Fig. 4.5A and 5B; W=3.0296, p=0.0818, W=3.0801, p=0.0793, respectively). Placental Pb was significantly associated with lower *5-HT$_{2A}$R/HTR2A* mRNA expression in the placenta (Fig. 4.5B; W=5.7278, p=0.0167). No significant difference was observed for 5-HT$_{2A}$R protein expression when more and less Mn or Pb were compared (Fig. 4.5C and 5D).

4.5. Discussion

This study suggests that low Pb and Mn concentration can alter the placental levels of 5-HTR et SERT. This is, to our knowledge, the first study evaluating the effects of metals on the placental 5-HT$_{2A}$R and SERT.

Exposure to Pb, even at low concentrations, decreased the SERT protein expression in placenta and BeWo cells, but not the *SLC6A4* mRNA expression. This implies that Pb could alter the mRNA translation into proteins. In addition, specific SERT activity in BeWo cells was decreased by low concentrations of Pb and Mn. Intracellular calcium $[Ca^{2+}]_i$ concentration can modulate SERT protein expression and activity via calmoduline (Seimandi *et al.*, 2013). Since Pb and Mn can interact with $[Ca^{2+}]_i$ (ATSDR, 2004b, Hong *et al.*, 2014, Lafond *et al.*, 2004), the decrease in SERT expression and activity could occur via a perturbation of $[Ca^{2+}]_i$ homoeostasis. Moreover, the affinity of metals for diverse functional groups found on proteins such as sulfhydryl group (Disbudak *et al.*, 2002, Zawia *et al.*, 2000) could perturb SERT functions, since SERT binding sites have an affinity for cations (Rudnick, 2006). Thus, divalent metals such as Pb and Mn may block 5-HT transport. More studies are needed to understand the toxicological mechanism of

metals on SERT expression and activity. It should be pertinent to include interaction effects between Pb and Mn and also between other essential metals.

5-HT levels in medium did not change, even though they should increase since 5-HT its transport by SERT is less effective. However, as expected, 5-HT levels in BeWo cells decreased after Mn or Pb exposure. A decrease in 5-HT levels was also observed in the brain of rats exposed to Pb (Widmer *et al.*, 1991) and to Mn (Bonilla *et al.*, 1984). Moreover, Mn exposure is associated with a decrease in levels of 5-hydroxyindoleacetic acid (5-HIAA), the main 5-HT metabolite (Takser *et al.*, 2003). The latter could be explained by an increased MAO activity, but our study does not support this hypothesis, as well as studies of Abdelouahab *et al.* (2010) and Subhash *et al.* (1991). Thus, the 5-HIAA level reductions are more likely to be linked to an overall decrease in 5-HT levels.

In light of the above, Mn and Pb could alter TPH activity, the limiting enzyme in 5-HT production, which led to less 5-HT. Khan *et al.* (2000) demonstrated that Pb decreased TPH activity in Atlantic croaker. Mn also decreased TPH activity in humans (Ogawa *et al.*, 2006). A study including the effect of metals on placental TPH could enlighten how Mn and Pb disturb 5-HT levels. Moreover, a perturbation of available L-tryptophan for 5-HT synthesis could also explain the lower 5-HT levels. L-tryptophan is involved in the kynurenine pathway through the indoleamine 2,3-dioxygenase (IDO), which the activity is increased by prostaglandins synthesized by cyclooxygenase (COX) (Muller *et al.*, 2007). However, in our study, this pathway seems not involved, since COX activity was not affected by Mn and Pb exposure (Supplementary data; Fig. 4.S3). Moreover, Goeden *et al.* (2016) recently showed, in mice, that inflammation result in an unregulated placental tryptophan conversion to 5-HT in the placenta, leading to an increased of 5-HT.

Lower SERT activity and lower 5-HT production could decrease the 5-HT levels in the cord blood since it is suggested that mother and placental 5-HT is essential in early fetus development (Bonnin *et al.*, 2011a, Côté *et al.*, 2007, Côté *et al.*, 2003, St-Pierre *et al.*, 2015). Lower 5-HT could, among others, alter the heart and brain development (Bonnin *et al.*, 2011a, Noorlander *et al.*, 2008, Sari *et al.*, 2003). Thus, deregulation in 5-HT system in placentas and fetus could lead to lifelong health problems by fetal programming (Holloway *et al.*, 2015, St-Pierre *et al.*, 2015). The placental tissue study also demonstrated that Mn and Pb can decrease *5-HT$_{2A}$R/HTR2A* mRNA expression. Our group demonstrated that DOI (2,5-demethoxy-4-indoamphetamine), a specific agonist of 5-HT$_{2A}$R, increased cell viability, proliferation, and migration in choriocarcinoma cell lines (Arseneault *et al.*, 2005, Oufkir *et al.*, 2010). Thus, a

perturbation of the receptor could alter placental growth. However, 5-HT_{2A}R protein expression was not different between higher and lower concentrations, neither in BeWo cells. Since BeWo cells come from a choriocarcinoma, they could act differently from healthy cells. However, they allow a better understanding of individual contaminant effects. Placental tissues, on the other hand, could be exposed to other contaminants than those studied, which could have additive, potentiation or synergetic effects on the *HTR2A* mRNA expression. Adding a primary cytotrophoblast cell culture isolated from placentas at delivery and exposed to Mn and Pb afterward could confirm results our study.

Mn and Pb concentrations found in the placental tissues study and used in the *in vitro* study were similar to those previously reported in maternal blood, cord blood and placenta of unexposed women (Abdelouahab *et al.*, 2010, Kim *et al.*, 2015, Osman *et al.*, 2000, Smargiassi *et al.*, 2002, Thomas *et al.*, 2015). In Canada, the Federal–Provincial Committee on Environmental and Occupational Health recommend an action for high blood Pb level contamination beginning at 10 µg/dL (480 nM) (Health Canada, 1994). This concentration seems very high compared those found in our study and the geometric mean Pb concentration in the general Canadian population (1.3 µg/dL = 63 nM) (Health Canada, 2015). Health Canada recently released a report underling the fact that Pb concentrations in blood lower than 10 µL/dL are associated with serious health effects, especially on infant and child development (Health Canada, 2013).

Because Mn is an essential metal, it is challenging to legislate a guideline limit since a deficiency or excess can impair the health of the mother or fetus (Goldhaber, 2003, Mergler, 1999). Moreover, Mn in maternal blood increases during pregnancy and is actively transported into the cord blood (Takser *et al.*, 2004a, Tholin *et al.*, 1995) demonstrating a crucial role for development. In fact, Mn concentrations were higher in the placenta than blood (our study and Osman *et al.* (2000)). This accumulation could be a way to stock the essential metal in case of deficiency or, in contrary, could be a defence to protect the fetus of effects caused by high concentrations.

Considering all the explanations and results above, and Mn and Pb effects on fetal and child development and health previously described (Bouchard *et al.*, 2011, Grandjean *et al.*, 2006, Neal *et al.*, 2013), it appears that a retrospective study focussing on the impact of an *in utero* exposure to Mn and Pb on the placental 5-HT system should be conducted to link 5-HT system alterations to children’s mental health problems.

4.6. Conclusion

Using a trophoblast model and a placental tissue study, this research demonstrated that Mn and Pb alter the placental 5-HT system at low concentrations.

Since 5-HT is implicated in the placental and fetal development, the modifications observed in this study may alter the pregnancy well-being and the fetus health. Further investigations are needed in order to better understand the 5-HT system roles in the placenta and the effects of environmental contamination. The study also put an emphasis on the importance of studying low environmental concentrations of contaminants since high concentrations cannot always predict effects observed. Future studies should include all 5-HT components, especially the expression and the activity of TPHs. It could be interesting to follow child development and health to evaluate the impact of prenatal 5-HT system perturbations over time.

4.7. Acknowledgments

The authors would like to thank Valentin Debard and Lise Parent (Télé-Université, Canada) for their contribution. This work was supported financially by the Discovery and Strategic Grant of Natural Sciences and Engineering Research Council of Canada to CV (NSERC: no 03948-2014), the Toxic Substances Research Initiative of Health Canada to JL and DM, and by studentship awards from the *Fonds de recherche du Québec nature et technologies*, the *Fondation universitaire Armand-Frappier* INRS, and the Canadian Institutes of Health Research (CIHR) Emerging Team Grant entitled Gender, Environment and Health (GTA92108) to MF, and the *Fondation universitaire Armand-Frappier* INRS, and the Canadian Institutes of Health Research (CIHR) to MV.

4.8. Figures

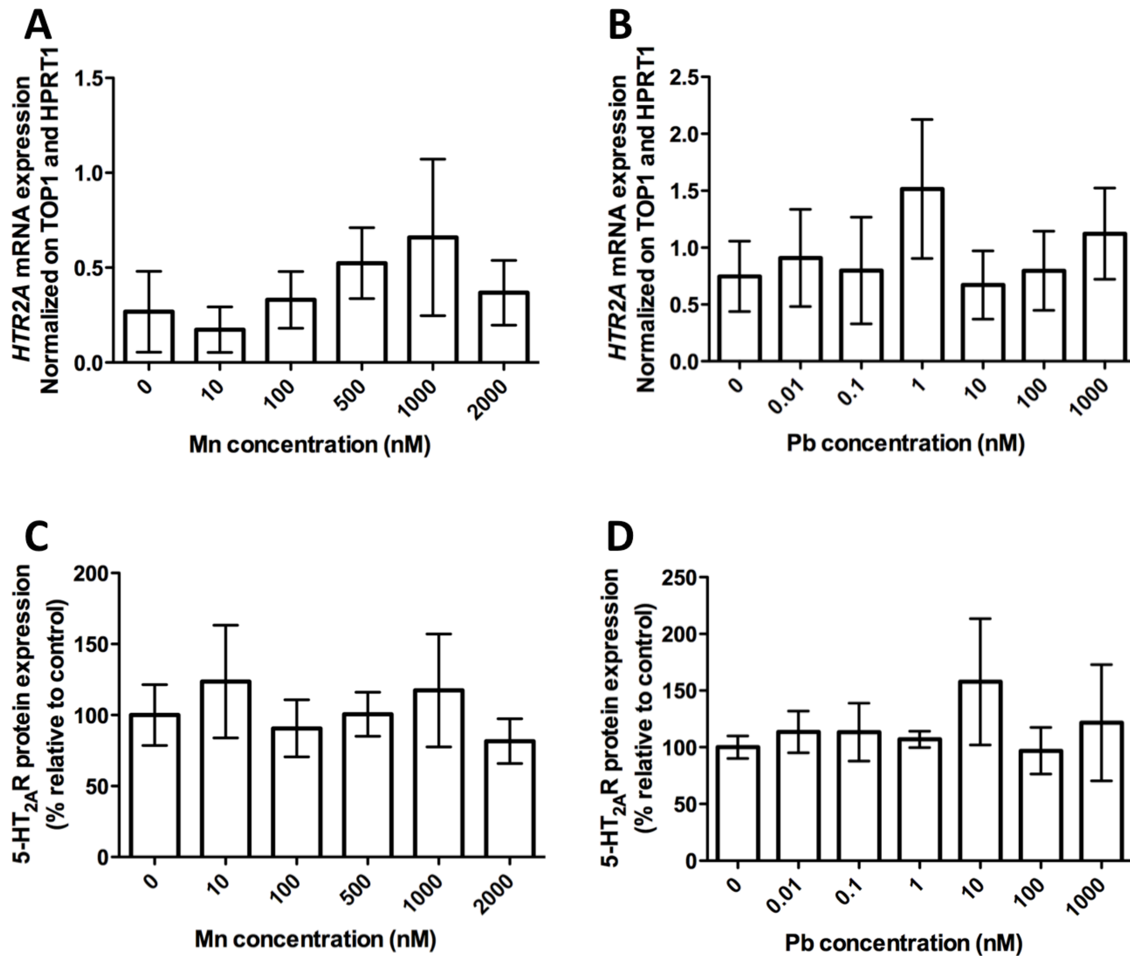

Figure 4.1: 5HT$_{2A}$R and *HTR2A* expression in BeWo cells exposed to Mn and Pb

Cells were exposed 24 h to low environmental concentrations of Mn (A and C) and Pb (B and D). *HTR2A* mRNA expressions (A and B) were normalized on *TOP1* and *HPRT1* mRNA expression. 5-HT$_{2A}$R protein expressions were normalized on control expression (0 nM). All results are expressed as mean ± SEM. No significant difference was observed (Kruskal-Wallis or Wilcoxon test, $P>0.05$).

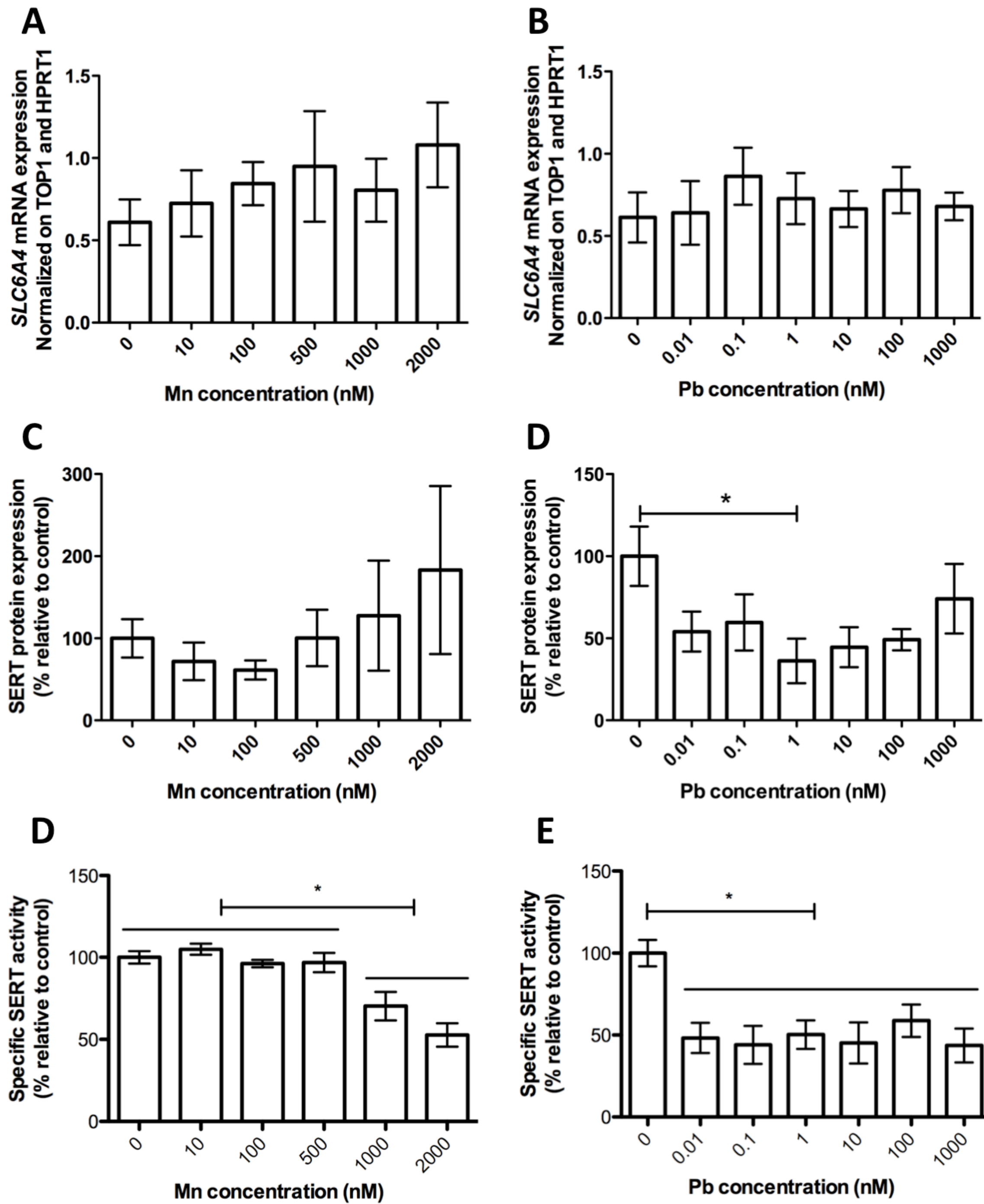

Figure 4.2: SERT and *SCL6A4* expression and specific SERT activity in BeWo cells exposed to Mn and Pb

Cells were exposed 24 h to low environmental concentrations of Mn (A and C) and Pb (B and D). *SCL6A4* mRNA expressions (A and B) were normalized on *TOP1* and *HPRT1* mRNA expression. SERT protein expressions were normalized on control expression (0 nM). For specific activity, cells were exposed 2 h to Mn (E) and Pb (F). Results were normalized on control activity (0 nM). All results are expressed as mean ± SEM. Asterisk (*) indicates a significant difference compared to control (0 nM) (Kruskal-Wallis or Wilcoxon test, P <0.05).

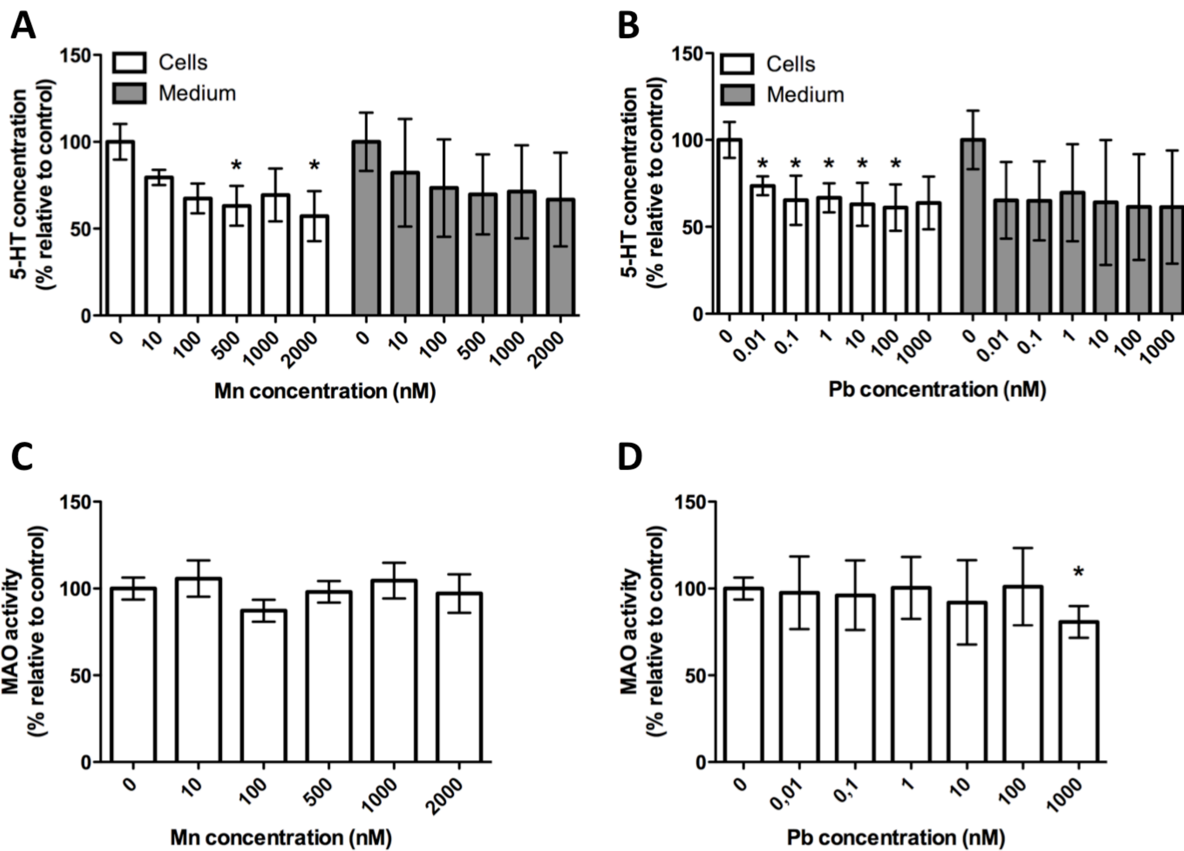

Figure 4.3: Serotonin levels (A and B) and MAO activity (C and D) in BeWo cells exposed to Mn and Pb

Cells were exposed 2 h to low environmental concentrations of Mn (A and C) and Pb (B and D). Results were normalized on control (0 nM) and are expressed as mean ± SEM. Asterisk (*) indicates a significant difference compared to control (0 nM) (Wilcoxon test, P <0.05).

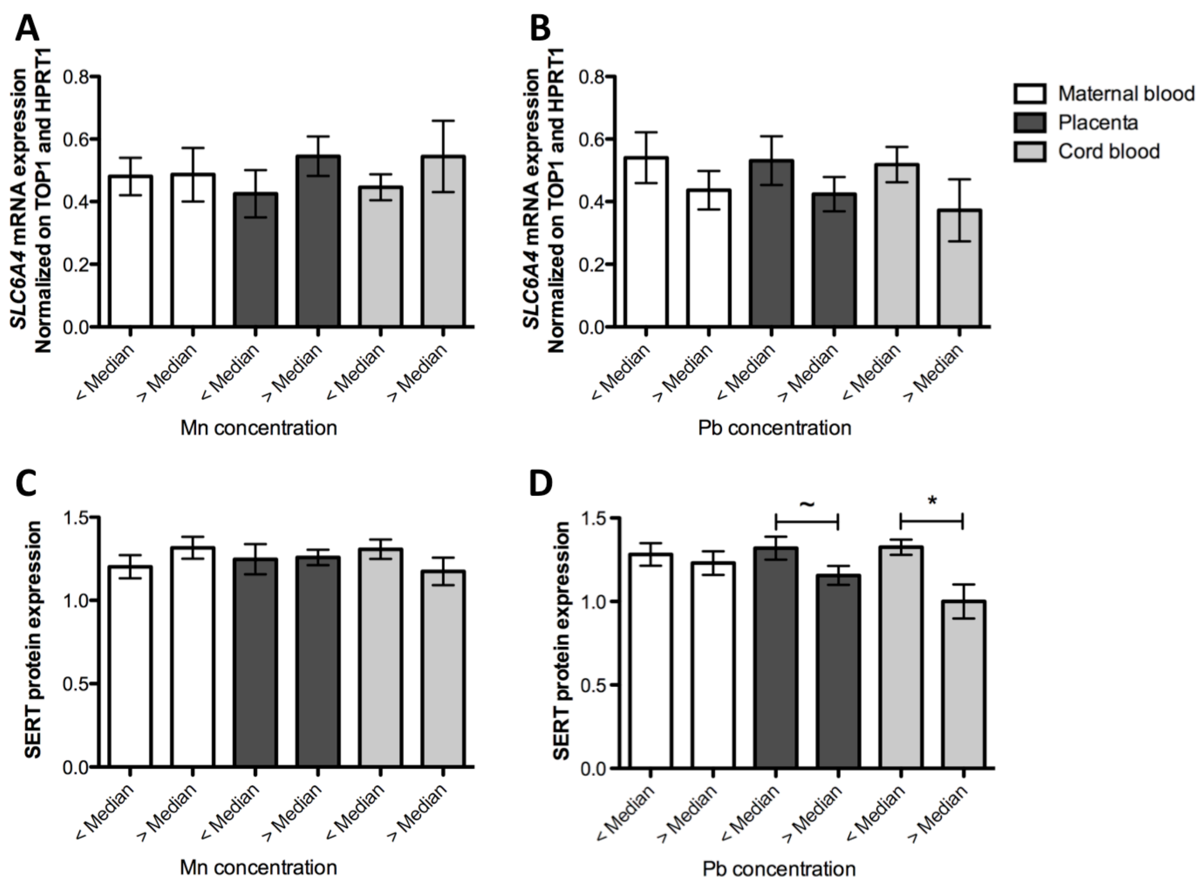

Figure 4.4: *SLC6A4* and SERT and expression in placentas according to Mn (A and C) and Pb (B and D) concentrations in maternal blood, placenta, and cord blood at delivery

SLC6A4 mRNA expression (A and B) was corrected for TSH concentration in maternal blood at delivery and normalized on *TBP* expression. SERT protein expression was corrected for TSH concentration in maternal blood during the 2nd trimester and for gestational age. All results are expressed as mean ± SEM. Asterisk (*) indicates a significant difference between less and more Mn or Pb fixed by median concentrations (*P <0.05). Tilde (~) shows a tendency that did not reach the significance threshold (Wilcoxon test, 0.1> P>0.050)

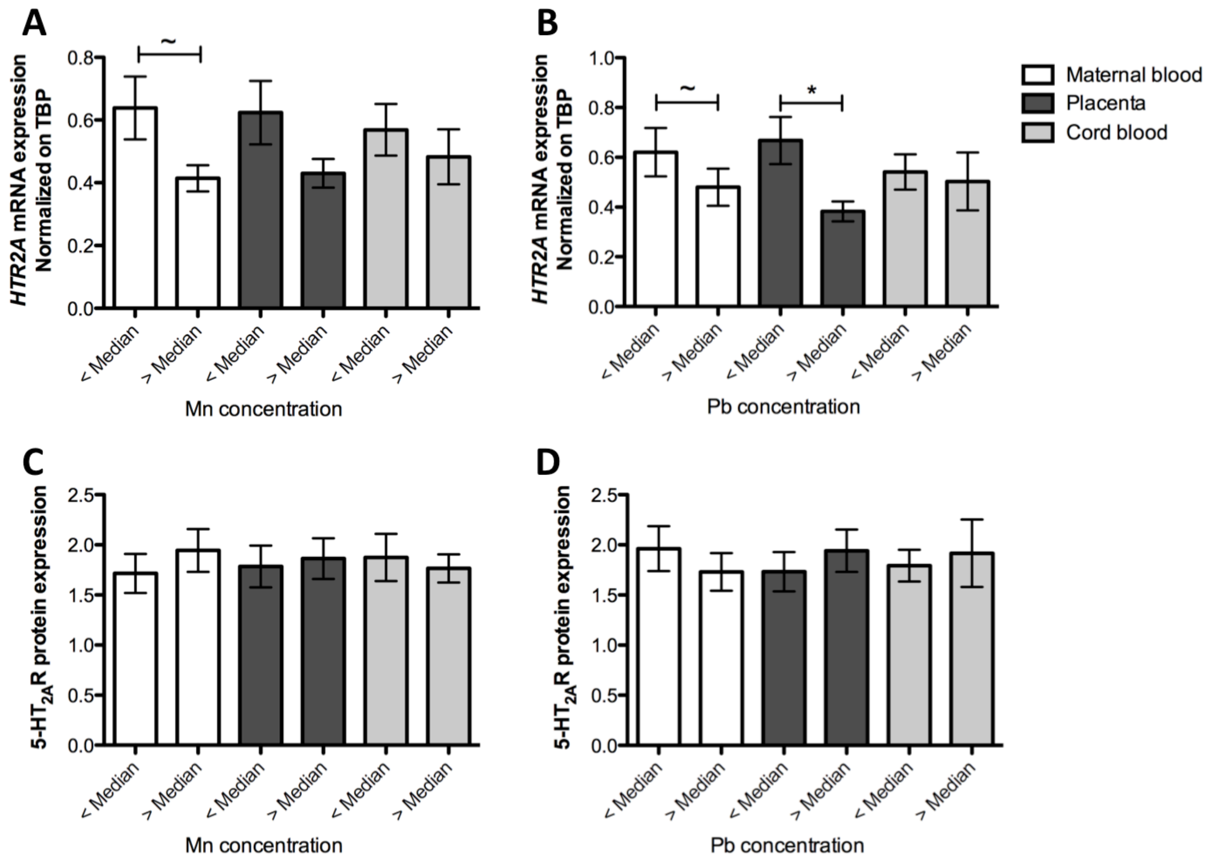

Figure 4.5: *HTR2A* and $5HT_{2A}R$ expression in placentas according to Mn (A and C) and Pb (B and D) concentrations in maternal blood, placenta, and cord blood at delivery

HTR2A mRNA expression (A and B) was corrected for maternal age and maternal weight gain and normalized on TBP expression. $5HT_{2A}R$ protein expression was corrected for maternal weight gain. All results are expressed as mean ± SEM. Asterisk (*) indicates a significant difference between less and more Mn or Pb fixed by median concentrations (*P <0.05). Tilde (~) shows a tendency that did not reach the significance threshold (Wilcoxon test, 0.1> P>0.050)

4.9. Supplementary data

4.9.1. Methods

4.9.1.1. RT-qPCR

For *in vitro* study, extracted RNA concentration and purity were measured by spectrophotometry (nanoDrop 1000 spectrophotometer, Thermo Fisher Scientific, MA, USA) before being stored at -80°C until analysis. Only RNA with an OD 260/280 ratio of 1.8 to 2.0 was used for further analysis. Quality of RNA was checked in random samples determined by electrophoresis through agarose gel stained with ethidium bromide. Reverse transcription reaction was done on 0.5 µg of RNA with the iScript cDNA synthesis kit for qPCR (Bio-Rad, ON, Canada) followed by a target-specific preamplification using 2.5 µL of cDNAs with the SsoAdvanced PreAmp Supermix (Bio-Rad, ON, Canada). PCR reactions were performed on a CFX-96 using SsoAdvanced Universal SYBR Green supermix (Bio-Rad, ON, Canada). Specific primers for *5-HT$_{2A}$R/HTR2A* and *SERT/SCL6A4* were designed and described by Viau *et al.* (2009) (Table 4.1). Seven reference genes were tested using GeNorm software (BioGazelle, Zwijnaarde, Belgium) (Lanoix *et al.*, 2012a, Vandesompele *et al.*, 2002). Hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and type 1 topoisomerase (*TOP1*) were selected for normalization (ΔΔCT).

For placental tissues, RNA purity and concentration were assessed by spectrophotometry at 260 and 280 nm (Spectramax, Molecular Devices Corporation, Sunnyvale, CA). RNA integrity was determined as described above.2 µg of each RNA sample were reverse-transcripted using Omniscript RT kit (Qiagen, ON, Canada). cDNA were stored at -20°C. Same specific primers, as described above, were used. Using the same protocol, TATA-binding protein (TBP) specific primers were designed as described above. The primer sequences are listed in Table 4.1. TBP RNA was used as a reference gene. PCR was performed on a LightCycler (Roche Diagnostics, QC, Canada) using LightCycler 480 SYBR Green I Master (Roche Diagnostics, QC, Canada). Normalized ratios were generated using the LightCycler 480 Relative Quantification Software (Roche Diagnostics, QC, Canada) using the ΔΔCT method.

4.9.1.2. Western Blots

Protein extraction in cell was done with radioimmunoprecipitation buffer (RIPA) containing protease inhibitors (cOmplet ULTRA Tablets, Roche, Laval, Canada) and a phosphatase inhibitor cocktail (Thermo Scientific, Burlington, Canada). After sonication, solutions were centrifuged (14,000 xg) at 4°C during 10 min. The supernatant were stored at -80°C until analysis.

After migration and bocking, membranes were incubated with mouse anti-SERT antibodies (1:1000) (ab181034, Abcam, Cambridge, MA) for 1h30 RT or with rabbit anti-5-HT_{2A}R *(1:250)* (sc-50396, Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. Membranes were then incubated with secondary anti-mouse or anti-rabbit antibody HRP conjugate (AP192P or AP307P; Millipore, Etobicoke, Canada) diluted 1:10,000 in 5% PBS skimmed milk for 1h at RT. The chemiluminescence was detected using Clarity Western ECL Blotting Substrate (Bio-Rad, ON, Canada) and revealed under Chemidoc MP imaging system (Bio-Rad, ON, Canada).

Protein extration in placental tissues was done as follow : blood compounds were eliminated by centrifugation with a lysis buffer containing 17mM Tris-HCl and 0.144 M NH4Cl, pH 7.3. The supernatants were mechanically homogenized with the Tissue Protein Extraction Reagent (T-PER, Pierce Biotechnology, Rockford, IL) supplemented with 1 µL phenylmethanesulfonyl fluoride (PMSF) in dimethyl sulfoxide (DMSO) per 1 mL T-PER and 2 µL Protease inhibitor cocktail (Sigma-Aldrich, ON, Canada) per 1 mL T-PER. Protein were stored at -80°C until analysis.

After migration and bocking, membranes were incubated with the mouse anti-SERT (1:1000, 2h at RT) (AB-N09, Advanced Targeting Systems, San Diego, CA), mouse anti-5-HT_{2A}R (1:500, ON at 4°C) (556326, BD Biosciences, ON, Canada) or mouse anti-GAPDH (1:500, 20 min at RT) (MAB374, Millipore, ON, Canada) primary antibody. This was followed 1h (or 20 min for GAPDH) incubation with secondary anti-mouse or anti-rabbit IgG HRP-conjugated secondary antibodies (AP192P or AP307P; Millipore, Etobicoke, Canada) diluted 1:10,000. Immunoreactive proteins were detected by autoradiography using Millipore Immobilon Western chemiluminescence kit (Millipore, ON, Canada). The chemiluminescence was detected using FluorChem and revealed under AlphaFC (Alpha Innotech, San Leandro, CA).

4.9.1.3. SERT activity

Cells were washed 3 times with PBS before the assay. A Krebs-Ringer's-HEPES medium (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \bullet 7H_2O$, 10 mM HEPES pH 7.4, 1.8 g/L glucose) containing phenelzine sulfate (100 µM) and ascorbic acid (100 µM) was used as an assay buffer. Cells were incubated 2h at 37°C in the assay buffer with or without Mn or Pb. All treatments were done in triplicate. Afterward, 5-HT uptake was initiated by adding an assay buffer containing 5 nM of tritium-labeled 5-HT (50 nM Hydroxytryptamine creatinine sulfate, 5-[1,2-^{3}H(N)], 1 µCi (PerkinElmer, Boston, MA)). After 20 min, cells were quickly washed 3 times on ice with cold PBS and detached in RIPA (200 µL). 100 µL of each well were transfered in a 24-well flexible microplate. A scintillation cocktail (Thermo Fisher Scientific, MA, USA) was added. Accumulated radioactivity was directly quantified in a microplate scintillation counter MicroBeta TriLux (PerkinElmer, Boston, MA). Specific [3H]-5-HT uptake was measured by subtracting the radioactivity accumulated in the presence of 50 µM fluoxetine, a selective serotonin reuptake inhibitor. The experiment was repeated at least three times. Protein concentrations were determined as previously described for each well. Results were normalized on control for each repetition.

4.9.1.4. Monoamine oxidase activity

The reaction mixture consisted of 100 µM tyramine, 1 µM dichlorofluorescein diacetate, 100 µM aminotriazole and 20 µg/mL horseradish peroxidase in 10mM HEPES pH 7.4 containing 140 mM NaCl. The reaction was incubated for 60 min at 30°C and fluorescence readings were measured at 485/20 nm excitation and 528/20 nm emission every 3 minutes

4.9.2. Tables

Table 4.S1: TSH concentrations (mIU/L) in maternal blood during pregnancy and cord blood at delivery
Determined by radioimmunoassay as described by Forest et al. (1998) at the Clinical Biochemistry Service of Saint-François d'Assise Hospital (QC, Canada).

	n	TSH concentration (mIU/L) *Mean ± SD (range)*
1st trimester	13	2.1 ± 1.2 (0.4 – 4.0)
2nd trimester	30	2.2 ± 1.0 (0.3 – 4.5)
At delivery	30	2.7 ± 1.5 (1.1 – 7.1)
Cord blood	29	13 ± 10 (4.6 – 52)

Table 4.S2 : Spearman correlations between metal concentrations in biological samples
* represent a statistical difference (p<0.05)

			Mn			**Pb**		
			Maternal	Placenta	Cord	Maternal	Placenta	Cord
Mn	Maternal	ρ	1	0.075	.532*	0.157	0.097	0.121
		p		0.714	0.009	0.444	0.639	0.573
	Placenta	ρ	0.075	1	0.216	-0.052	0.331	.475*
		p	0.714		0.299	0.802	0.069	0.014
	Cord	ρ	.532*	0.216	1	0.173	0.293	0.339
		p	0.009	0.299		0.43	0.156	0.097
Pb	Maternal	ρ	0.157	-0.052	0.173	1	.668*	0.177
		p	0.444	0.802	0.43		<0.0001	0.407
	Placenta	ρ	0.097	0.331	0.293	.668*	1	.503*
		p	0.639	0.069	0.156	<0.0001		0.009
	Cord	ρ	0.121	.475*	0.339	0.177	.503*	1
		p	0.573	0.014	0.097	0.407	0.009	

4.9.3. Figures

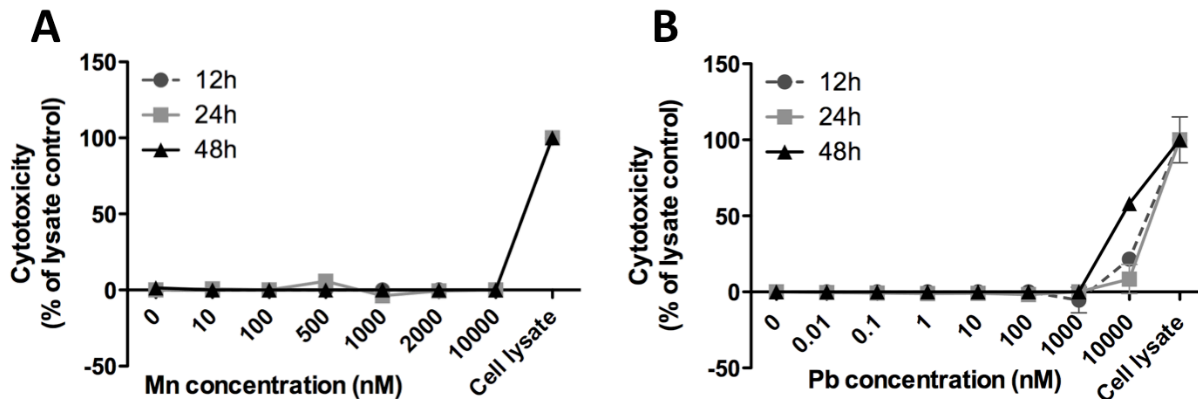

Figure 4.S1: Mn (A) and Pb (B) concentration used for BeWo cells exposure were not cytotoxic.

Cytotoxicity was determined by lactate dehydrogenase (LDH) activity assay (Roche, Basel, Switzerland) following the manufacturer's instructions. A high metal concentration (10,000 nM) were tested and cell lysate was used as positive control. The experiment was conducted after exposure of 12, 24 and 48 h of exposure. Results are expressed in percentage of cell lysate control.

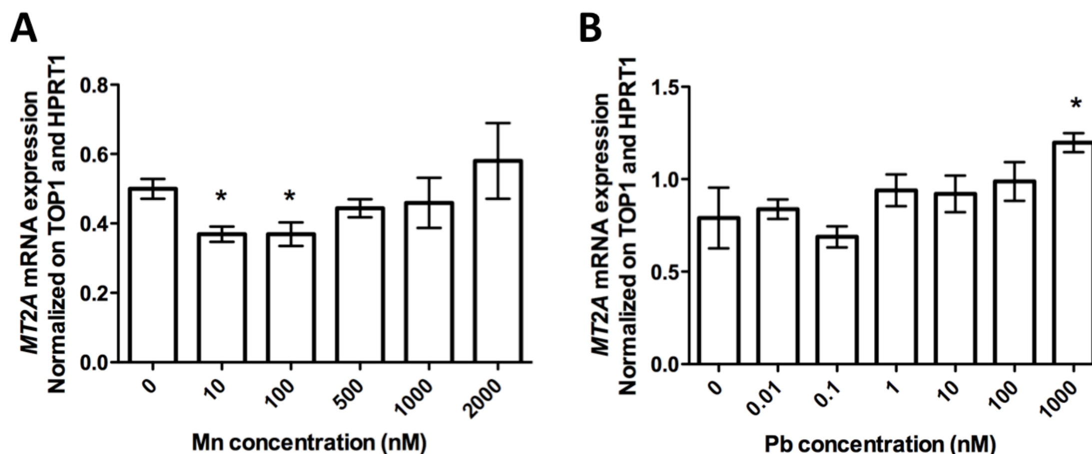

Figure 4.S2: Metallothionein 2A (*MT2A/NM-005953.3*) mRNA expression in BeWo cells exposed to Mn (A) and Pb (B)

BeWo *MT2A* mRNA expression decreased significantly compared to control when exposed 24 h to Mn concentrations (10 and 100 nM; both W=3.8571, p=0.0495) and increased when exposed 24h to 1000 nM Pb (W=3.8571, p=0.0495), showing that metal could reach cells.
RT-qPCR, with specific primers F-5'-GGGCGTCGGACAAGTG-3' and R-5'- GAATATAGCAAACGGTCACGG-3', was used as described in section 2.3. Product is 127 bd. Results were normalized on *TOP1* and *HPRT1* mRNA expression and are expressed as mean ± SEM. Asterisk (*) indicates a significant difference compared to control (0 nM) (Wilcoxon test, P<0.05).

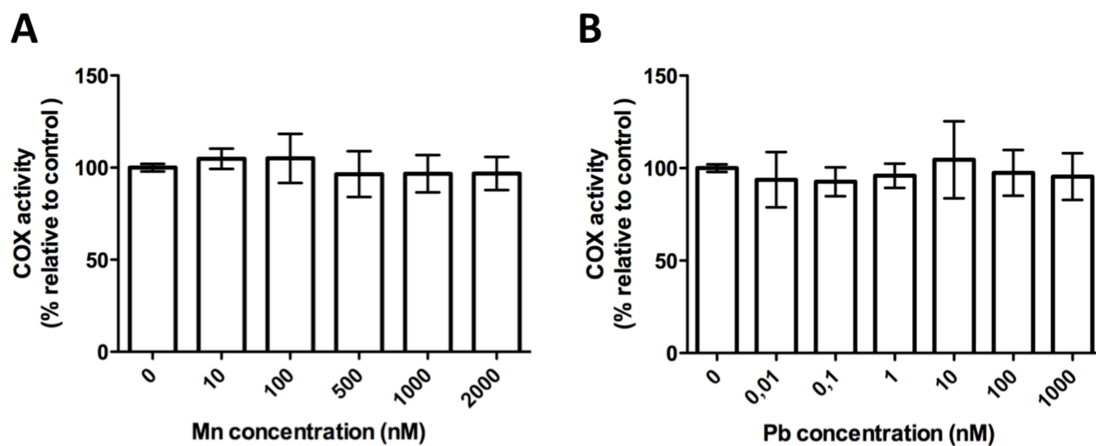

Figure 4.S3: Cyclooxygenase (COX) activity in BeWo cells exposed 2h to Mn (A) and Pb (B).

Metal exposures did not change BeWo COX activities (Mn: H=4.192, p=0.522; Pb: H=1.898, p=0.929), COX activity was measured as described by Gagné *et al.* (2011b). Results are normalized on control activity (0 nM) and are expressed as mean ± SEM. Asterisk (*) indicates a significant difference compared to control (0 nM) (Wilcoxon test, P<0.05).

CHAPITRE 5 :
DISCUSSION ET CONCLUSION GÉNÉRALE

Ce projet de recherche démontre que de faibles concentrations de Mn, Pb et Cd altèrent le système 5-HT chez *Mytilus edulis* et le trophoblaste placentaire humain (Tableau 5.1). Il s'agit de la première étude démontrant un effet des métaux sur le système 5-HT chez la moule bleue et le trophoblaste placentaire humain (Tableau 1.1). De plus, les concentrations de Mn, Pb et Cd utilisées dans le cadre des expositions sont plus faibles que celles utilisées dans les études antérieures et représentent des conditions environnementales (Tableau 1.1).

Tableau 5.1. : Tableau récapitulatif des résultats présentés dans cette thèse.
Effet du plomb (Pb), manganèse (Mn) et cadmium (Cd) sur le système sérotonine chez la moule bleue et le trophoblaste placentaire humain.

Modèles expérimentaux	*In vivo* (moule)			*In vitro* (BeWo)			*Tissus* (placenta)	
Métal / **Biomarqueurs**	**Mn**	**Pb**	**Cd**	**Mn**	**Pb**	**Cd**	**Mn**	**Pb**
5-HT – concentrations	–	–	–	↓	↓	↓		
MAO	–	–	↓	–	↓	–		
SERT – protéine	↓	↓	↓	–	↓	–	–	↓
SERT – activité				↓	↓	–		
SLC6A4 – ARNm				–	–	↓	–	–
HTR2A – ARNm				–	–	–	↓ (gris)	↓
$5HT_{2A}R$ – protéine				–	–	↑	–	–

↓Noir : Significatif (p <0,05) ↑Gris :Tendance (0,1>p>0,05) − : Pas d'effets (p >0,1)

La présente étude démontre que l'expression protéique du SERT est significativement diminuée suite à une exposition au Pb tant chez la moule bleu (mollusque) (> 0,01 nM) que le trophoblaste placentaire (humain) (BeWo : > 1 nM; sang cordon: > 50 nM; placenta : > 20 nM) (Chapitre 3 et 4; Tableau 5.1). Par contre, le Mn et le Cd diminuent l'expression protéique de SERT chez la moule, mais pas dans les cellules BeWo (Chapitre 3 et 4; Annexe 1). Les différences d'effets pourraient être causées par : i) une action des métaux qui diffère entre les modèles (moule et placenta humain), comme il a été observé entre le guppy et le poisson-zèbre exposés au Pb sur l'activité de la MAO (tableau 1.1) (Senatori *et al.*, 2009). ii) le mode

d'exposition (exposition long terme sur l'organisme entier pour les moules et exposition cellulaire directe pour les cellules BeWo). iii) une différence de conformation structurale de la protéine du SERT, malgré la grande conservation du gène *Scl6a4* entre les espèces (Caveney et al., 2006). En outre, des polymorphismes au niveau du promoteur *Scl6a4* montrent des différences d'activités du SERT (Homberg *et al.*, 2011, St-Pierre *et al.*, 2015). De plus, le variant court (S) du SERT conduit à une transcription 2 à 2,5 fois plus faible que le variant long (L), mais présente une activité similaire (Heils *et al.*, 1996, Nakamura *et al.*, 2000); [iv) Une différence de régulation de la dégradation de la protéine chez les moules vs l'humain et/ou dans la régulation de la traduction serait également possible. Cependant, un effet sur la transcription ne semble pas impliqué puisque l'expression de l'ARNm du *SCL6A4* n'est pas modifiée, suggérant plutôt un effet post-transcriptionnel. Ces hypothèses restent à vérifier pour bien cerner la différence entre les effets des métaux sur la moule bleue et le trophoblaste placentaire humain.

Le Mn, le Pb et le Cd sont connus pour leur interaction avec le Ca. Ils perturbent les mécanismes régulés par le Ca par une compétition pour les canaux calciques et les sites de liaisons au Ca sur les protéines. Par exemple, les métaux pourraient perturber le transport de la 5-HT via une altération avec la calmoduline, impliqué dans l'expression du SERT et modulée par les [Ca]i (ATSDR, 2004b, Jayanthi *et al.*, 1994, Lafond *et al.*, 2004, Seimandi *et al.*, 2013, Tchounwou *et al.*, 2012). L'activité du SERT dans les cellules BeWo est diminuée par une exposition aux concentrations faibles de Pb (≥ 0,01 nM) et de Mn (≥ 1000 nM), mais pas de Cd (Tableau 5.1). Salanki *et al.* (1990) démontrent qu'une exposition de l'anodonte des cygnes (*Anadonta cygea*) à 100 µM de Pb et de Cd diminue le transport de la 5-HT dans le ganglion pédal. Les métaux, en raison de leur affinité pour les groupements des protéines, tel que les thiols et les amines, pourraient perturber le fonctionnement de la protéine SERT en se liant à ses sites actifs ou en modifiant sa configuration (ATSDR, 2004b, Disbudak *et al.*, 2002, Rudnick, 2006, Zawia *et al.*, 2000). Par exemple, les cations sont susceptibles d'interagir avec les sites de liaisons du Na^+ ou du K^+ nécessaire au transport symport-antiport du SERT (Rudnick, 2006).

L'étude présentée au chapitre 3 démontre une possible interaction entre le Mn et le Pb. Dans les bassins d'expositions au Mn (100 et 1000 nM), des concentrations non négligeables de Pb ont été mesurées dans les eaux usées. Une étude antérieure de notre équipe a montré des concentrations plus faibles en Mn dans des sites industriels avec des concentrations élevées de Pb que dans les sites de références (Fraser *et al.*, 2011). De plus, une exposition à un mélange de Mn et Pb augmente le temps de demi-vie du Pb dans le sang des lapins (ATSDR, 2004b, Momoko *et al.*, 1984). Ceci suggère que le Mn et le Pb sont en compétitions pour les mêmes

sites de liaisons chez les moules, comme il a été démontré chez les lapins (Kalia *et al.*, 1984). Il serait intéressant de mener une étude d'interaction des métaux, y compris les métaux essentiels, tels que le Ca et le Zn, afin de mieux comprendre le mécanisme d'actions des métaux sur le système 5-HT. De plus, l'étude des effets d'une exposition mixtes aux contaminants inorganiques et organiques permettrait de mieux saisir les dynamiques d'interactions dans un contexte plus près de la réalité environnementale que des études contrôlées en laboratoire. De plus, l'eau commerciale utilisée comme véhicule et témoin au chapitre 3 contenait du Mn et Pb. Bien que l'interprétation des résultats ne soit pas altérée par ceci, l'analyse de la concentration en métaux dans les moules aurait été un ajout important afin d'établir les niveaux d'expositions aux métaux. Ceci aurait également pu permettre de confirmer si les métaux se sont rendus au moule et quelle portion du métal était assimilée.

Le chapitre 4 et l'annexe 1 montrent une diminution des concentrations de 5-HT dans les cellules BeWo à la suite d'une exposition au Pb, Mn et Cd (Tableau 5.1). Ce phénomène a également été décrit dans le cerveau du rat où une diminution des concentrations de 5-HT en réponse à une exposition au Pb, Cd ou Mn a été observée (Tableau 1.1) (Bonilla *et al.*, 1984, Nation *et al.*, 1989, Widmer *et al.*, 1991). Cependant, chez les moules exposées à ces trois métaux pendant 28 jours, la concentration de 5-HT n'est pas modifiée (Tableau 5.1). Il est possible que les taux de 5-HT aient été altérés par les premiers temps d'exposition, mais que les effets se soient rétablis compte tenu de la longue exposition (28 jours), comme il a été démontré chez la moule brune (*Perna perna*) et l'anodonte des cygnes (*Anadonta cygnea*) exposés 120 h (Tableau 1.1) (Almeida *et al.*, 2003, Salanki *et al.*, 1990). Une étude de cinétique permettrait de mieux cerner la régulation du transport et de la production de la 5-HT dans la moule bleue.

Les chapitres 3 et 4 démontrent que l'activité de la MAO est diminuée par une exposition au Cd chez la moule bleue et une exposition au Pb chez les cellules BeWo (Tableau 5.1). La littérature demeure controversée quant aux effets du Pb et du Cd sur l'activité de la MAO (Tableau 1.1). Une exposition au Pb du tambour brésilien (*Micropogonias undulatus*) induit une augmentation de l'activité de la MAO dans l'hypothalamus (Khan *et al.*, 2000). En revanche, une exposition au Pb cause une diminution de l'activité de la MAO dans un homogénat de cerveau du poisson-zèbre (*Danio rerio*) (Senatori *et al.*, 2009). Il est possible que le Pb présente un effet différent sur l'une ou l'autre des isoformes de la MAO (MAO-A et MAO-B). En effet, l'isoforme prédominant peut être différent en fonction de l'organe ou de l'espèce. Une exposition au Cd chez les planaires (*Dugesia japonica*) induit une diminution de l'activité de la MAO-B et une

augmentation de l'activité de la MAO-A dans la tête (Tableau 1.1) (Wu *et al.*, 2015). La méthode utilisée pour mesurer l'activité de la MAO dans la présente étude ne permet pas de discriminer les isoformes puisque la tyramine n'est pas un substrat spécifique (Youdim *et al.*, 2004). L'utilisation de substrat ayant des affinités spécifiques aux différentes isoformes de la MAO permettrait de discriminer les effets des contaminants sur chacune des isoformes (Finberg, 2014). De plus, l'expression et l'activité des isoformes de la MAO varient selon l'organe dans lequel on les retrouve (Billett, 2004). Dans le placenta, l'isoforme A est sept fois plus active que l'isoforme B (Billett, 2004). La diminution de l'activité de la MAO observée dans les BeWo exposées au Pb est probablement due à une perturbation de l'isoforme A. Pour vérifier ceci, une étude incluant des inhibiteurs spécifiques des isoformes de la MAO pourrait être effectuée.

L'étude populationnelle pilote présentée au chapitre 4 démontre que les placentas associés contenant des concentrations de Mn et de Pb plus élevées dans le sang maternel et contenant plus de Pb dans le placenta ont une plus faible expression de l'ARNm *HTR2A* sans modifier l'expression protéique. Cette observation n'est pas retrouvée dans l'étude *in vitro* avec les cellules BeWo (Chapitre 4). Cette différence rappelle que les cellules BeWo dérivent d'un choriocarcinome de placenta humain, et représentent donc un modèle « *trophoblast-like* » des primocultures. De ce fait, les BeWo peuvent agir de manière différente que les cellules saines, quoiqu'elles présentent des caractéristiques similaires. De plus, le tissu placentaire est plus complexe comprenant l'ensemble des types cellulaires (ex. : trophoblaste, endothélial…) qui peuvent interagir lors d'une exposition à des contaminants. Ces études devront être répétés avec des primocultures de cellules cytotrophoblastiques ou sur des explants isolés à partir de placentas humains de grossesse normale afin de confirmer ou infirmer les effets des métaux sur le système 5-HT observées sur le tissu et les cellules BeWos. Les effets du Pb, Cd et Mn sur le récepteur 5-HT_{2A} chez la moule bleue n'ont pas pu être déterminés dans le cadre de cette thèse, puisqu'aucun anticorps contre la protéine n'est disponible et que la séquence génique n'est pas connue. Cependant, puisque la présence d'un récepteur 5-HT_{2A}-like est suggérée, il sera important d'analyser celui-ci dans le futur (Fong *et al.*, 2003).

Une attention particulière a été portée sur les différences d'effets entre les sexes étant donné que les systèmes physiologiques sont différents entre les mâles et les femelles (pour revue Ritz *et al.* (2014)). De plus, il a été montré que le système 5-HT et les effets des métaux peuvent différer selon le sexe (Abdelouahab *et al.*, 2010, Linder *et al.*, 2011, Montes *et al.*, 2008, Wang *et al.*, 2010). Dans l'étude populationnelle, aucun effet sexe-spécifique n'est observé. Une étude à plus grande échelle incluant plus de femmes enceintes permettrait de déterminer avec plus de

pouvoir statistique si le sexe du fœtus influence les effets des métaux sur le système 5-HT placentaire. La mise en place d'un protocole efficace de détermination des sexes chez la moule (Chapitre 1) a permis une étude comparative des effets des métaux sur le système 5-HT de manière sexe-spécifique (Chapitre 3). L'étude montre que l'activité de la MAO est plus élevée chez les femelles que les mâles dans le bassin témoin (Chapitre 2). Ceci peut s'expliquer par le fait que le gène codant pour la *MAO* se situe sur le chromosome X (Pintar *et al.*, 1981). En effet, des études ont montré que l'activité de la MAO est aussi plus élevée chez les femmes que chez les hommes (Murphy *et al.*, 1976, Robinson *et al.*, 1971, Sandler *et al.*, 1981). Étant donné qu'il était seulement possible de déterminer le nombre de moules de chaque sexe à la fin de la période d'exposition, le ratio mâle/femelle de chacune des conditions expérimentales variait beaucoup, réduisant ainsi le pouvoir statistique. L'utilisation d'un nombre plus élevé d'effectifs chez les moules (*in vivo*) aurait pu augmenter le pouvoir statistique nécessaire à l'étude comparée des sexes.

À la lumière des résultats présentés dans cette thèse, le système 5-HT apparaît comme un paramètre d'effet d'intérêt qui pourrait être intégré dans d'autres études portant sur les effets des contaminants. Une étude des effets des effluents des eaux usées de la communauté urbaine de la ville de Montréal sur le système 5-HT de la mulette de l'est (*Elliptio complanata*) abonde ce sens (Annexe 2). Les eaux usées contiennent un mélange de nombreux contaminants, dont des métaux et des antidépresseurs (Gagné *et al.*, 2001, Gagnon *et al.*, 2006, Lajeunesse *et al.*, 2011). Dans les gonades des moules exposées aux effluents, l'expression du de la protéine SERT est diminuée de près de 50 % et les taux de 5-HT et l'activité MAO sont augmentés d'environ 20 % (Annexe 2). Ceci suggère une activité sérotoninergique des effluents. Des études supplémentaires avec d'autres contaminants, tels que des produits pharmaceutiques serait intéressantes étant donné qu'ils sont également présents dans l'environnement (Gagné *et al.*, 2001, Gagnon *et al.*, 2006, Lajeunesse *et al.*, 2011).

En conclusion, ce projet de recherche a permis d'étudier trois modèles d'étude non invasifs, représentatifs de leur environnement et facilement accessibles pour déterminer les effets des métaux sur le système 5-HT. Ces travaux ont permis de démontrer que de faibles concentrations de Mn, un métal essentiel, de Pb et de Cd, des métaux toxiques, altèrent le système 5-HT des moules bleues et du trophoblaste placentaire humain. La poursuite de cette étude permettra, entre autres, de déterminer l'effet des métaux sur d'autres paramètres du système de la 5-HT, tels que les TPH, afin de mieux cerner les effets de ces contaminants sur le

système 5-HT. Puisque la 5-HT impliquée dans les mécanismes de reproduction chez la moule bleue et dans le bien-être de la grossesse chez l'humain, les modifications observées dans le système 5-HT pourraient avoir des répercussions sur les populations de moules ainsi que sur le développement du fœtus. La figure 6.1 propose un modèle de perturbation de la reproduction chez l'humain et la moule par les métaux via le système 5-HT. D'autres études sont nécessaires pour cerner le mécanisme impliqué dans les effets des métaux sur le système sérotoninergique, et si les effets sont similaires interespèces et interorganes. Dans des travaux menés en parallèle de cette thèse, nous montrons que le système immunitaire des moules est altéré pendant la ponte (Annexe 3.1), les rendant ainsi plus vulnérables aux contaminants environnementaux (Annexe 3.2). De plus, la 5-HT est impliquée dans la différenciation sexuelle, la gamétogénèse et la ponte (Aiello, 1965; Fong et al., 2003; Gibbons and Castagna, 1984; Gies, 1986; Ram et al., 1999). Il serait donc intéressant de poursuive ces études afin de déterminer les effets des métaux sur le système 5-HT aux différents stades du cycle de reproduction chez la moule bleue, puisque les effets pourraient varier de ceux obtenus dans la présente étude où, les moules étaient toutes en période post-ponte. Enfin, une étude prospective à long terme associant les effets d'une exposition des métaux sur le système 5-HT placentaire et les répercussions sur la grossesse et le développement cognitif des enfants permettrait de comprendre si le une perturbation du système 5-HT est impliqué dans les perturbations du développement associées à une exposition in utero aux métaux (Grandjean *et al.*, 1997, Hu *et al.*, 2006, Kummu *et al.*, 2012).

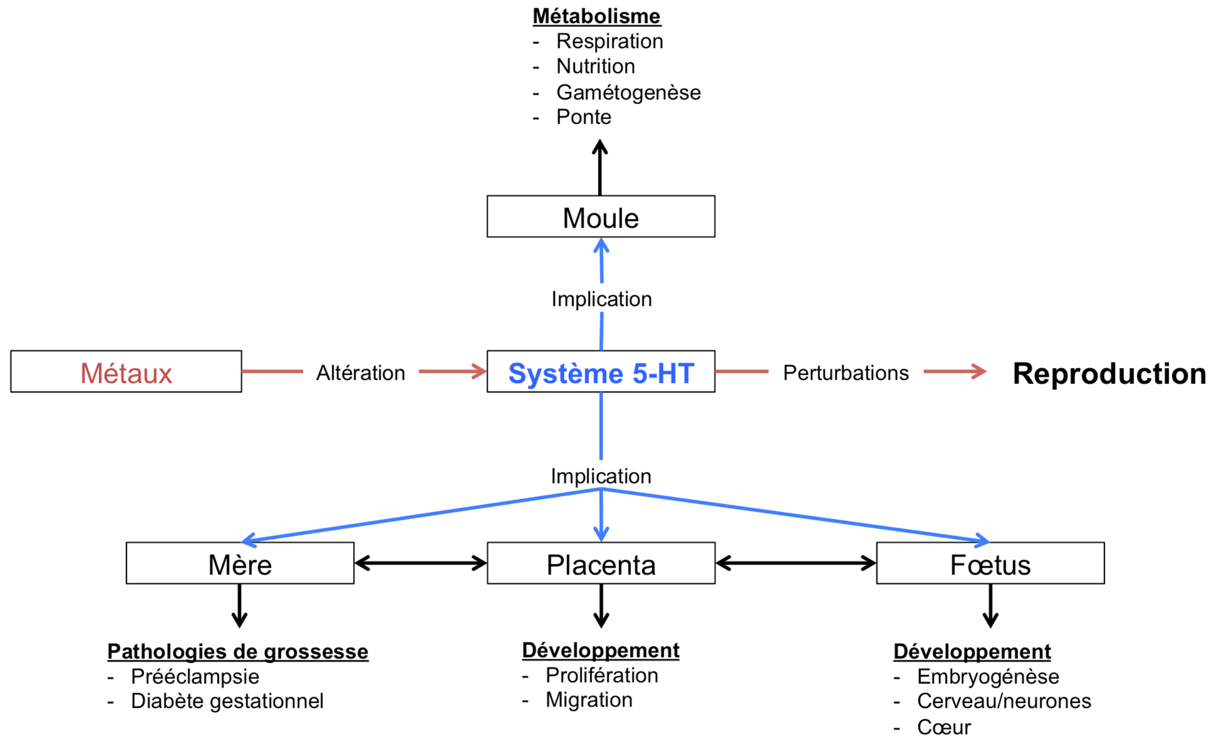

Figure 5.1 : Modèle proposé d'une perturbation des fonctions reproductrices chez l'humain et la moule par les métaux via une altération du système 5-HT
Une altération des composantes du système 5-HT par le Mn, le Pb et le Cd peut perturber les fonctions de la sérotonine chez la moule et chez l'humain au cours de la grossesse.

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ANNEXE 1 :
EFFECT OF CADMIUM ON SEROTONIN SYSTEM IN BEWO CELLS

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Contribution de l'étudiant

L'étudiant a participé à l'élaboration de l'étude, réalisé toutes les expériences et analysé les résultats dans cet article. Il a également rédigé l'article et participé au choix du journal de publication.

Résumé de l'article en français

Le système 5-HT se retrouve dans le placenta et est indispensable au bon déroulement de la grossesse, aux fonctions placentaires et au développement fœtal. Une exposition environnementale maternelle aux métaux peut interférer avec les fonctions placentaires et perturber le développement embryonnaire. Le Cd perturbe le système 5-HT chez certains animaux. Cependant, l'effet du cadmium sur le système 5-HT placentaire humain n'a jamais été étudié. Les cellules BeWo ont été exposées à des concentrations croissantes de Cd (0,1 à 2000 nM). L'exposition au Cd a diminué la concentration de 5-HT et l'expression de l'ARNm codant *SERT/SCL6A4*, alors que l'expression de la protéine 5-HT_{2A}R a été augmentée. Cette étude montre que de faibles concentrations de Cd interfèrent avec le système 5-HT, ce qui pourrait nuire à la santé de la grossesse et au développement fœtal.

EFFECT OF CADMIUM ON SEROTONIN SYSTEM IN BEWO CELLS

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A1.1. Abstract

Serotonin system is found in the placenta and is essential for healthy pregnancy, placental function, and fetal development. Maternal environmental exposure to metals may interfere with placental functions and disturb embryonic development. In animals, cadmium disturbs the serotonin system. Cadmium effects on the trophoblast serotonin system have never been studied. BeWo cells were exposed to increasing cadmium concentrations (0.1 to 2000 nM). Cadmium decreased serotonin levels and serotonin transporter mRNA expression, whereas the serotonin receptor 2A protein expression increased. This study shows that low cadmium concentration interfere with the trophoblast 5-HT system, which could impair pregnancy health and fetal development.

Keywords

Serotonin transporter, metal, serotonin receptor 2A, placenta, trophoblast

A1.2. Introduction

Cadmium (Cd), highly present in cigarettes, is a toxic metal that can lead to serious health problems and protein malfunctions (Jarup, 2003, Kummu *et al.*, 2012). During pregnancy, maternal Cd can reach and accumulate in the placenta, and also cross its barrier (Esteban-Vasallo *et al.*, 2012). This exposure can disturb placental functions and fetal growth and health (Erboga *et al.*, 2015, Vilahur *et al.*, 2015, Ward *et al.*, 1987).

Cd can disturb the serotonin (5-HT) system in different animal models (Almeida *et al.*, 2003, Lafuente *et al.*, 2001, Salanki *et al.*, 1990, Wu *et al.*, 2015). This monoamine, acting as a neurotransmitter, a growth factor, and a hormone, is synthesized from L-tryptophan by tryptophan hydroxylase enzymes (TPH) (Jonnakuty *et al.*, 2008). 5-HT count many receptors, including 5-HT_{2A}R. 5-HT transport in the cells occurs by its transporter (SERT) where it could be metabolized by monoamine oxidases (MAO) (Jonnakuty *et al.*, 2008). Serotonin system is expressed in the placenta and it is contributing to its development (Arseneault *et al.*, 2005, Bonnin *et al.*, 2011a, Deroy *et al.*, 2013, Oufkir *et al.*, 2010, Sonier *et al.*, 2005, Viau *et al.*, 2009). Moreover, it is suggested that maternal and placental 5-HT, transiting through placental SERT, is essential in early fetus heart and brain development (Bonnin *et al.*, 2011a, Côté *et al.*, 2007, St-Pierre *et al.*, 2015). Thus, a 5-HT system disruption in the placenta by Cd could lead to pregnancy and fetal health problems (St-Pierre *et al.*, 2015). However, effects of Cd on the human placental 5-HT system have never been studied. This study aimed to evaluate the effects of low environmental Cd concentration on the 5-HT system of the BeWo choriocarcinoma cell line, a model of the human trophoblast.

A1.3. Methods

BeWo cells (ATTC, Manassas, VA) were exposed 2 or 24 h, as described before (Chapitre 4), to $CdCl_2$ (0.1 to 2000 nM) (Sigma-Aldrich, ON, Canada) in order to reflect placental and blood Cd levels found in previous studies (Esteban-Vasallo *et al.*, 2012, Thomas *et al.*, 2015). Western blotting (SERT and 5-HT_{2A}R), reverse transcriptase real-time PCR (*SERT/SCL6A4*, *5-HT2AR/HTR2A* and metallotionein (*MT2A*)), and SERT, MAO, and COX activity assays were performed as described elsewhere (Chapitre 4). Lactate dehydrogenase (LDH) activity assay (Roche, Basel, Switzerland) and Serotonin ELISA kit (IBL, Germany) were performed following the manufacturer's instructions as described elsewhere (Chapitre 4). Nonparametric Kruskal-Wallis tests were conducted to compare all metal concentrations. When a tendency was

suspected in results, Wilcoxon tests were applied to compare specific concentrations with control and a Benjamini-Hochberg correction was applied. Statistics were computed with SPSS 23.0 (IBM corp., 2015). P <0.05 was considered statistically significant.

A1.4. Results and Discussion

This study is a first attempt to show the effects of Cd on the 5-HT system of the human placenta. Cd concentrations were not cytotoxic and did not cause inflammation via COX activity (Fig. A1.1A and A1.1C). A metallothionein (MT) induction occurred for 100, 1000, and 2000 nM of Cd (Fig. A1.1B), suggesting a protection mechanism (Nakamura *et al.*, 2012). However, it should be determine if *MT2A* mRNA increased is reflected in an increased of MT protein expression. In the placenta of non-exposed women, Cd concentration are often less than 100 nM (Esteban-Vasallo *et al.*, 2012). However, in women exposed, such as smokers, Cd placental concentrations may exceed 1000 nM (Esteban-Vasallo *et al.*, 2012, Ronco *et al.*, 2005), which are associated with higher MT expressions (Ronco *et al.*, 2005).

5-HT concentrations decreased in cells exposed to 2000 nM Cd (Fig. A1.2A). Studies on molluscs exposed to similar Cd concentrations showed the same patterns (Almeida *et al.*, 2003, Salanki *et al.*, 1990). An increased MAO activity could explain this effect on 5-HT concentration, but it was not the case in this study (Fig. A1.2B). MAO-A activity in freshwater planarian tails was not disrupted after a Cd exposure of 4 days, but was decreased after 7 days (Wu *et al.*, 2015). A longer exposure might have shown an effect on MAO activity in this study.

5-HT concentration decrease could be explained by higher SERT activity. Nevertheless, *SERT/SCL6A4* mRNA expression decrease after an exposure to 1000 nM Cd (Fig. A1.2C). Cd is well known for its interaction with zinc (Brzoska *et al.*, 2001). Thus, Cd could perturb zinc finger proteins, transcription factors, leading to mRNA perturbation (Green *et al.*, 1998). However, *$5\text{-}HT_{2A}R/HTR2A$* mRNA expression was not affected by Cd, but its protein expression increased at 2000 nM (Fig. A1.2F). SERT protein expression was not altered by Cd (Fig. A1.2D). Low Cd concentrations can alter the function of other important placental transporters in BeWo (Kummu *et al.*, 2012), suggesting that SERT activity may be disturbed. However, SERT activity was not affected by Cd in our study (Fig A1.2E). Those results suggest that the slight decreased in 5-HT concentration may be due to a TPH perturbation.

SERT activity and 5-HT level alterations by Cd could disrupt fetus growth, such as heart and brain development, since it is suggested that maternal and placental 5-HT is essential (Bonnin *et*

al., 2011a, Côté *et al.*, 2007). Moreover, 5-HT_{2A}R is implicated in viability, proliferation, and migration of placental cells (Arseneault *et al.*, 2005, Oufkir *et al.*, 2010, Sonier *et al.*, 2005). Thus, a perturbation of the receptor could alter the placental growth.

Other studies are needed to understand if Cd alters the placental 5-HT system. Those studies should include: 1) TPH expression and activity; 2) other models such as explant and primary cell culture of placentas; and 3) more time points to follow the kinetic action of Cd. This is important since a deregulation of the 5-HT system in the placenta and fetus could lead to lifelong health problems (St-Pierre *et al.*, 2015, Vilahur *et al.*, 2015).

A1.5. Acknowledgments

Authors would like to thank Valentin Debard. This work was supported financially by the discovery and strategic grant of Natural Sciences and Engineering Research Council of Canada to CV (NSERC: no 03948-2014) and by studentship awards from Fonds de recherche du Québec (FRQ)-Nature et technologies (NT), Fondation universitaire Armand-Frappier INRS, and Canadian Institutes of Health Research (CIHR) Emerging Team Grant entitled Gender, Environment and Health (GTA92108) to Fraser, M.

A1.6. Figures

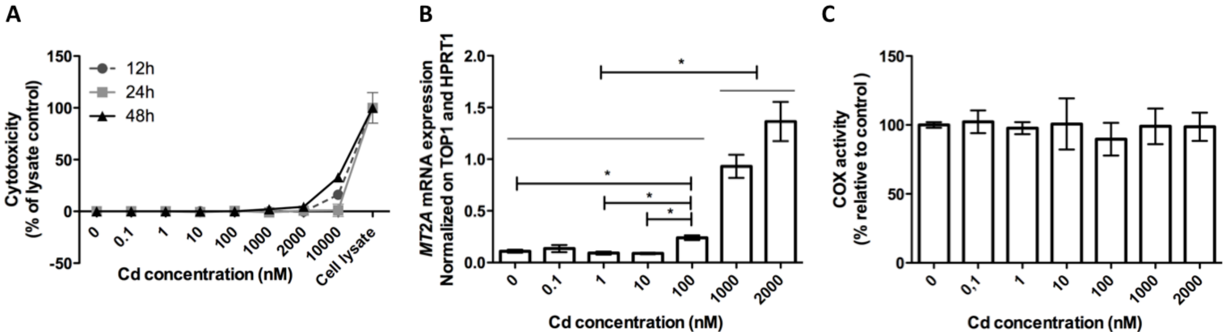

Figure A1.1: Effect biomarkers in BeWo cells treated with low Cd concentrations.

A] LDH activity released from the cytosol of damaged cells into the medium after exposure of 12, 24, and 48 h. Results are expressed in percentage of cell lysate control. **B]** Metallothioneine 2A mRNA expression after exposure of 24 h. Results were normalized on *TOP1* and *HPRT1* mRNA expression and are expressed as mean ± SEM. * represents a significant difference between exposure (Kruskall-Wallis, H=21.665, p=0.001). **C]** COX after 2h of exposition. Results were normalized on control activity (0 nM) and are expressed as mean ± SEM.

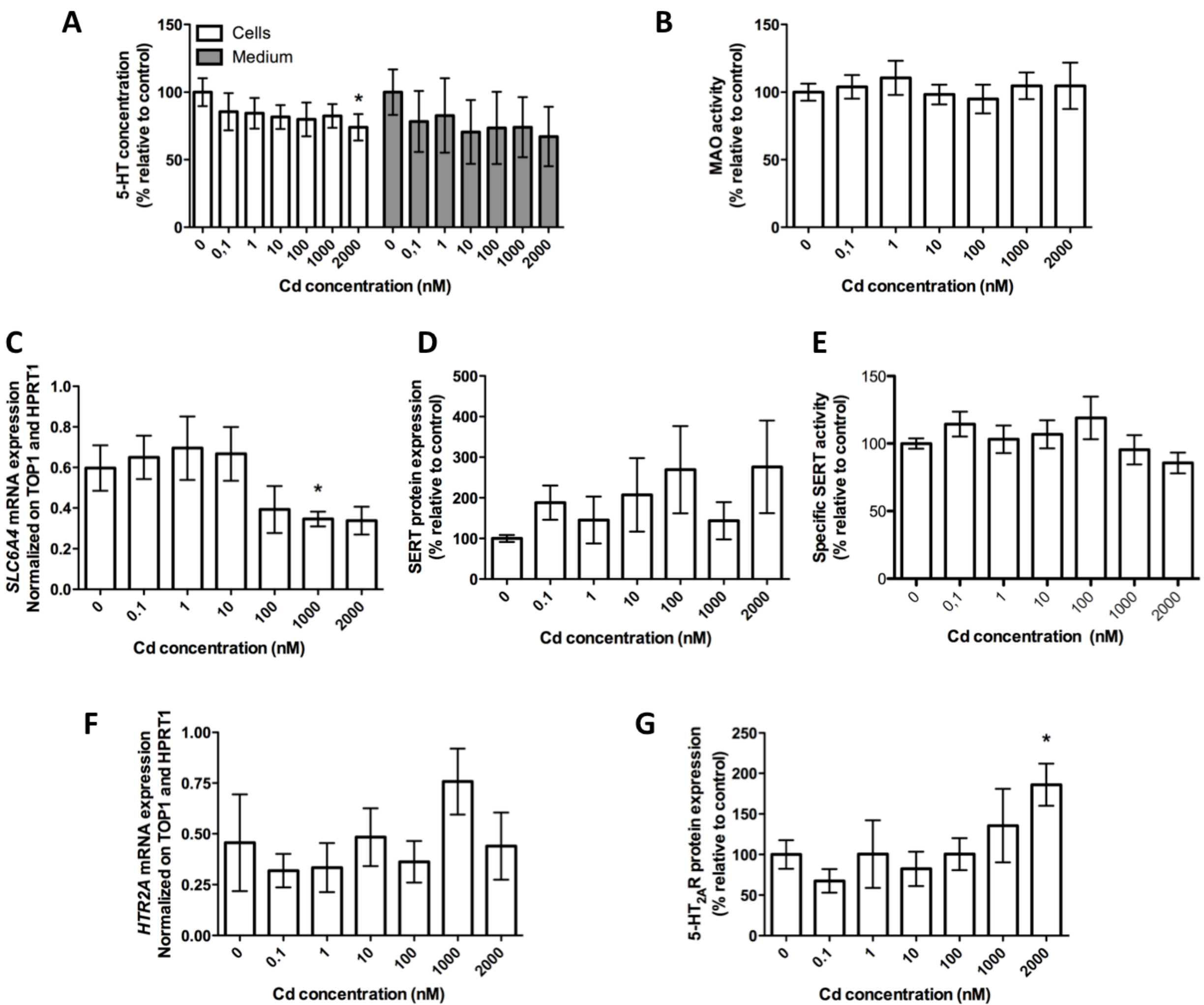

Figure A1.2: Low Cd concentrations had no significant effect on the BeWo cells serotonin system.

BeWo cells were exposed to increasing Cd concentration for 2h (A, B, and E) or 24 h (C, D, F and G). Serotonin (5-HT) levels in BeWo cells or medium (**A**), MAO activity (**B**), specific SERT activity (**E**), and SERT and 5HT2AR protein expression (**D** and **G**, respectively) were normalized on control (0 nM). *SERT/SCL6A4* and *5-HT$_{2A}$R/HTR2A* mRNA expressions (**C** and **F**, respectively) were normalized on *TOP1* and *HPRT1* mRNA expression. All results are expressed as mean ± SEM. * indicates a significant difference compared to control (0 nM) (Wilcoxon test, P <0.05, N=3-5).

ANNEXE 2 : EXPOSITION AUX EFFLUENTS DES EAUX USÉES DE LA VILLE DE MONTRÉAL : ALTÉRATION DU SYSTÈME SÉROTONINERGIQUE CHEZ LA MULETTE COMMUNE DE L'EST (*ELLIPTIO COMPLANATA*)

A2.1: Résumé

Les effluents des eaux usées des municipalités sont connus pour déverser différents produits pharmaceutiques dans l'environnement aquatique. Une accumulation des inhibiteurs sélectifs de la recapture de la sérotonine (ISRS) dans les moules exposées à l`effluent de la station d'épuration de la communauté urbaine de Montréal (CUM) indique la biodisponibilité de ces médicaments. Malgré l'importance de la sérotonine (5-HT) pour la reproduction et la survie de la moule, les effets des effluents d'eaux usées sur le système 5-HT ont peu été étudiés. L'objectif est de déterminer l'effet d'une exposition aux effluents des eaux usées de la CUM sur le système 5-HT de la mulette de l'est (*Elliptio complanata*). Les moules (n = 32) ont été exposées pendant 21 jours à des concentrations croissantes (0, 3, 10 et 20 %; n = 8 par réservoir) d'effluents d'eaux usées de la CUM. L'expression du transporteur de la sérotonine (SERT), les taux de 5-HT, l'activité de la monoamine-oxydase (MAO) et les concentrations d'ISRSs ont été déterminés dans les gonades. Les résultats montrent une corrélation positive entre les concentrations d'ISRSs dans les effluents et dans les moules exposées. Dans les gonades des moules exposées aux effluents, l'expression de SERT est diminuée d'environ 50 % et les taux de 5-HT et de l'activité MAO sont augmentés d'environ 20 %. Ces travaux montrent que les ISRSs présents dans les effluents des eaux usées de la CUM perturbent le système 5-HT des mulettes de l'est et, par conséquent, suggèrent une perturbation de la reproduction et du développement embryonnaire.

Contribution de l'étudiant

L'étudiant a déterminé l'expression de SERT, réalisé les analyses, interprété les résultats et préparé l'affiche qui a été présentée au congrès ÉcoBIM 2013, à la 1ère journée annuelle de recherche sur l'environnement du Regroupement en toxicologie, chimie et génie environnemental de l'Université du Québec et au colloque annuel du Chapitre Saint-Laurent.

A2.2 : Affiche

ANNEXE 3 :
AUTRES ARTICLES SCIENTIFIQUES PUBLIÉS

Deux articles hors objectifs ont été publiés par l'étudiant lors de ce doctorat. Ceux-ci sont issus de travaux entamés par Pablo Rault, stagiaire de l'équipe du Dr Michel Fournier, mais terminés par l'étudiant.

Contribution de l'étudiant

L'étudiant a contribué à l'élaboration de l'étude, réalisé les expériences sur le manteau des moules (dissections, LPO, COX et VTG) et analysé les résultats de l'article présenté en A2.1. Il a également interprété les résultats et rédigé l'article publié en 2013 dans le Journal of Xenobiotics (Fraser *et al.*, 2013).

L'étudiant a réalisé les analyses, interprété les résultats et rédigé l'article publié présenté en A2.2. L'article a été publié en 2014 dans le Journal of Xenobiotics (Fraser *et al.*, 2014).

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- Fraser M, Rault P, Fortier M, Brousseau P, Fournier M, Surette C & Vaillancourt C (2014) Immune response of blue mussels (Mytilus edulis) in spawning period following exposure to metals. *Journal of Xenobiotics* 4(4895):65-67. **DOI:** 10.4081/xeno.2013.s1.e12
- Fraser M, Rault P, Roumier P-H, Fortier M, André C, Brousseau P, Gagné F, Fournier M, Surette C & Vaillancourt C (2013) Decrease in phagocytosis capacity of hemocyte during spawning in Mytilus edulis: a pilot study. *Journal of Xenobiotics* 3(S1)(e12):31-33. **DOI:** 10.4081/xeno.2014.4895

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Journal of Xenobiotics 2013; volume 3(s1):e12

Decrease in phagocytosis capacity of hemocyte during spawning in *Mytilus edulis*: a pilot study

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Key words: phagocytosis, *Mytilus edulis.*

Conference presentation: part of this paper was presented at the *ECOBIM meeting*, 2013 May, Montréal, Quebec, Canada.

Contributions: Michel Fournier, CS, CV contributed equally.

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Introduction

During spawning in bivalves, important physiological systems are modulated. For example, an increase in serotonin (5-HT), a decrease of dopamine and an increase in the activity of cyclooxygenase (COX) are associated with spawning.[1-5] In *Elliptio complanata*, females produce eggs containing egg-yolk proteins rich in vitellogenin (Vtg), and levels of these proteins decrease during the spawning period once eggs are released.[6] In the Pacific oyster (*Crassostrea gigas*), it has also been shown that the organism reduces its metabolic activity after spawning due to a decrease in energy reserves.[7,8] Moreover, a decrease in hematocyte phagocytosis, which plays a central role in bivalve defense, was observed during spawning in the mantle of Pacific oyster.[7-10] A seasonal decrease, corresponding to the spawning period, of hemocyte phagocytosis was also featured in blue mussels (*Mytilus edulis*) from the Gulf of St. Lawrence.[11,12] Despite this observation, it has not been clearly demonstrated that the decrease in phagocytosis in *Mytilus edulis* is due, among other things, to spawning. Thus the aim of this pilot study is to determine the effect of spawning on the immune response (phagocytosis) of *Mytilus edulis*.

Materials and Methods

Animals

Mytilus edulis were collected from the *Baie de Plaisance* located in the *Îles de la Madeleine* region (47°29'N, 61°87'W). The specimens were placed in aerated tank filled with artificial sea water (Instant Ocean®, Reef Crystal, Cincinnati, OH, USA) (15ºC, salinity 31-1psu) and fed three times a week with phytoplankton (Phytoplex®, Kent Marine, Franklin, WI, USA). Mussels were sacrificed one week before (n=12) and during (n=11) spawning period. The spawning period was determined by the release of gametes in the water.

Gonads and hemolymph collection

Before dissection, hemolymph was collected individually from the posterior adductor muscle using a 3 mL syringe and used immediately for phagocytosis and cell viability assays. For biomarkers measurement, mussels were kept in 20°C. The mantles, containing gonads, were dissected on ice and homogenized with a Teflon pestle tissue grinder in a conservation buffer (Hepes-NaOH buffer (pH 7.4) containing 100 mM NaCl, 0.1 mM dithiothreitol and 1 µg mL-1 aprotinin) as described in *Gagné et al.*[13] The cytoplasmic fraction was obtained from a subsample of the homogenate as described in Gagné *et al.*[6] Total proteins were determined using the Bradford method.[14]

Gonads integrity

Lipid peroxidation in mantle homogenates was determined using the thiobarbituric acid method which reacts with malonaldehyde following the oxidative breakdown of unsaturated phospholipids.[15] Standards of tetramethoxypropane used for calibration and blanks were prepared in homogenization buffer. Fluorescence was measured at 540/35 excitation and 600/40 nm emission and results were expressed as thiobarbituric acid reactants/mg proteins.

Spawning biomarker

COX activity was measured as described by Gagné *et al.*[6,13] using the oxidation of 2,7-dichlorofluorescin in the presence of arachidonate, previously described in Fijimoto *et al.*[16,17] Briefly, S15 fraction were mixed with arachidonate, dichlorofluorescin and horseradish peroxidase in a Tris-HCL buffer (pH 8) containing Tween 20. The appearance of fluorescein was measured every 2 min for 20 min at 485 nm (excitation) and 520 nm (emission) with a spectrofluorimeter (Spectra Max M5, Molecular devices). Results are expressed in relative fluorescence units/min/mg proteins. The levels of Vtg-like proteins were determined by the alkali-labile phosphate principle in acetone-fractionated proteins in the S15 fraction in order to determine if the mussels have spawned.[18] Results are expressed in µg of phosphate/mg proteins.

Cell viability and phagocytosis of hemocytes

The viability of hemocytes was evaluated by flow cytometry using the Guava PCA flow cytometer (Guava Technologies, Hayward, CA, USA) and the Viacount kit (Guava technologies) according to the supplier's instructions. Briefly, an aliquot of hemolymph was mixed with Viacount and 1000 events were recorded. Phagocytosis was assessed by determining by flow cytometry according to Brousseau *et al.*[19] Briefly, hemocytes were mixed with yellow-green latex Fluoresbrite TM Carboxylate microspheres (Polysciences, Inc., Warrington, PA, USA), and incubated at 16°C in the dark. Additional beads were removed by centrifugation on a bovine serum albumin gradient. Acquisitions were performed using a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) equipped with a 488 nm argon laser. For each sample, the fluorescence of 10,000 events was recorded in the region of interest.

Statistical analysis

Data are presented as mean standard error of the mean. The results were tested for normality by the Kolmogorov-Smirnov test. Significant differences were determined using Student's t-test. Variance homogeneity was verified by Levene's test. Pearson's correlations were performed between biomarkers and phagocytosis capacity. SPSS 20.0 was used for all statistical analyses (IBM corp, 2011).

Results and Discussion

Lipid peroxidation levels showed that gonad integrity was similar before and during spawning period (P=0.825, Figure 1A). For mussels

still in the spawning period, no significant difference was observed in Vtg-like expression (P=0.471, Figure 1B). This occurrence could be due to the proximity of the mussels living in the same aquarium. Eggs released from one mussel into the water could be recaptured by the same mussel or another. Moreover, mussels should have been sexed by histological analysis of gonads in order to distinguish the females in our Vtg expression results, as these proteins are generally found only in eggs. In bivalves, COX activity increases during spawning it was not statistically significant": (P=0.160; Figure 1C).[1-3] In this study, COX activity seemed to increase even though it was not statistically significant. It is possible that some mussels sacrificed in the spawning period were still not in spawning process. However, the negative correlation between COX activity and level of Vtg in the gonads (r= 0.530; P=0.009; Table 1) gave a good indication that the mussels were, in fact, in spawning period. Moreover, phagocytosis activity in hemocytes was significantly and negatively correlated to COX activity in gonads (r= 0.413; P=0.05; Table 1), which supports the hypothesis of the interaction of spawning on phagocytosis activity. Future experiments should include histological analysis of gonads during gametogenesis and the analysis of other spawning biomarkers, like serotonin and dopamine levels.

Hemocyte viability was not altered by the spawning period (P=0.111; Figure 2). Non-spawning mussels showed a phagocytic capacity of 44% (Figure 2). Spawning induced a significant decrease of phagocytosis to reach 19% (P<0.009; Figure 2).

The decreased in immune response observed during spawning in *Mytilus edulis* in this study is consistent with the observation made by Cartier *et al*.[12] They observed a decrease of phagocytosis in mussels from the Gulf of St. Lawrence corresponding to the spawning period.[12] However, they were not able to make a direct link between those events. Nevertheless, a decrease in hematocyte phagocytosis was observed during spawning of *Crassostrea gigas*,[7-10] which is consistent with this study. Li *et al.* show that energy reserves, based on glycogen and protein levels in the mantle of Pacific oysters, are also decreased during the spawning period.[7] Thus, a trade-off of the energy to the spawning process could explain the effects on the immune response. This possibility should be tested for *Mytilus edulis*. Since the decrease of phagocytotic capacity occurs during spawning, it is also possible that the increased level of sex steroid hormones plays a suppressive role on the immune system, as was suggested by Cartier *et al.*[12,20] This phenomena was already demonstrated by Watanuki *et al.* in the head kidney of common carp.[21] It would be interesting to monitor different hormones, like progesterone and estradiol, before, during and after spawning period of *Mytilus edulis*. Li *et al.* observed a recovery of phagocytotic capacity 8 days after the spawning in Pacific oysters.[7] The same observation was made in *Mytilus edulis* after 7 days (data not shown). It would be useful to study extensively the impact of spawning on the immune system. During a period of reduced immune activity, it could be possible that mussels are more vulnerable to diseases.[7-9,12]

In conclusion, this preliminary study shows for the first time that spawning activity can be linked with a decreased in phagocytotic capacity in *Mytilus edulis*. A more complete study will be done based on the results presented here. It will be important to include histological analysis of the gonads in order to determine the gamatogenisis stage of each mussel. The impact of spawning should be considered in immunological studies in *Mytilus edulis*.

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Table 1. Pearson's correlation between phagocytosis capacity of hemocytes and biomarkers (COX and Vtg) in *Mytilus edulis*.

		Phagocytosis capacity	COX	Vtg
Phagocytosis capacity	r	1	–0.413*	–0.060
	Sig. (2-tailed)	1	0.050	0.787
COX	r	–0.413*	1	–0.530**
	Sig. (2-tailed)	0.050	1	0.009
Vtg	r	–0.060	–0.530*	1
	Sig. (2-tailed)	787	0.009	1

COX, cyclooxygenase; Vtg, vitellogenin. *P<0.05, ** P<0.01.

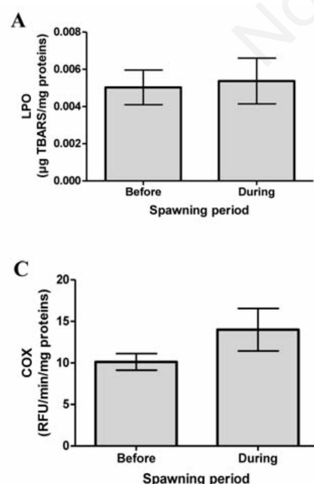

Figure 1. Analysis of (A) lipid peroxidation (LPO), (B) vitellogenin-like proteins and (C) in gonads of mussels before and during spawning period. No significant differences are observed. COX, cyclooxygenase.

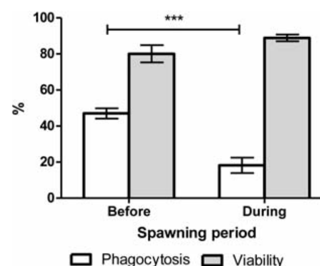

Figure 2. Phagocytic capacity and viability of hemocytes from *Mytilus edulis* before and during spawning period. ***P<0.0001.

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Immune response of blue mussels (*Mytilus edulis*) in spawning period following exposure to metals

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Introduction

Mytilus edulis are sensitive to environmental contamination like metals, such as silver (Ag), lead (Pb), cadmium (Cd) and mercury (Hg), which are bioavailable for this species.[1-3] It is well documented that these metals can disrupt the immune responses in bivalves.[4-6] During spawning, important physiological systems are involved, such as serotonin and dopamine,[7-11] which in turn could modulate the immune system.[12,13] Indeed, bivalves reduce their metabolic activity after spawning which is reflected by a decrease of the phagocytosis activity.[14-18] It is expected that during the period of reduced immune activity (*i.e.*, after spawning) mussels are more vulnerable to contaminations and diseases.[14-16,18,19] The aim of this pilot study was to determine if spawning modulates immune responses of *Mytilus edulis* exposed to metals (Ag, Cd, Hg and Pb).

Materials and Methods

Mussels

Mytilus edulis were collected from the *Baie de Plaisance* located in the *Îles de la Madeleine* region (QC, Canada) (47°29'N, 61°87'W), an area clear from industrial and urban activities. Upon arrival at the laboratory, the mussels were transferred and maintained in tank filled with artificial seawater (15°C, salinity 31-1 psu) and fed twice a week with a concentrate of phytoplankton (Phytoplex , Reef solution, Laval, QC, Canada). Mussels were sacrificed one week before (W-1) spawning, during (W0) spawning, two and three weeks after spawning period (W2 and W3 respectively). The spawning period was determined by the release of gametes in the water. Hemolymph was collected from the posterior adductor muscle of each mussel using a 3 mL syringe and immediately used for analysis.

Metal exposure

Mussel hemocytes were exposed *in vitro* to increasing metal concentrations (n=3 by metal): one metal at a time during 21 hours (Ag, Cd, Hg and Pb; 10^{-9} to 10^{-3} M). Metals used are reagent grade: cadmium chloride ($CdCl_2$), lead chloride ($PbCl_2$), mercuric chloride ($HgCl_2$) and silver nitrate ($AgNO_3$) (Sigma-Aldrich, ON, Canada).

Cell viability

The viability of hemocytes was evaluated by flow cytometry using the Guava PCA flow cytometer (Guava Technologies, CA, US) and the Viacount kit (Guava Technologies) according to the supplier's instructions. Briefly, an aliquot of hemolymph was mixed with Viacount and 1000 events were recorded.

Phagocytosis of hemocytes

Phagocytosis was assessed by flow cytometry according to Brousseau *et al.*[20] Briefly, hemocytes were incubated with latex fluorescent beads (Yellow-green Fluoresbrite, Polysciences®). The number of engulfed beads in each hemocyte was determined using a FACSCalibur (Becton-Dickinson, CA, USA) flow cytometer. Fluorescence emission was read in FL1 (λ=530 nm), with at least 10 000 events in the region of interest were recorded. Results were analyzed with the Cell Quest Pro software (Becton-Dickinson, CA, USA) to determine the percentage of hemocytes that engulfed one bead and more (phagocytic activity) or three beads and more (phagocytic efficiency).

Statistical analysis

Data are presented as mean standard error of the mean (SEM). The results were tested for normality by the Kolmogorov-Smirnov test. Variance homogeneity was verified by Levene's test. Significant differences were determined using ANOVA or Kruskal-Wallis. SPSS 20.0 was used for all statistical analyses (IBM Corp., 2011, IL, USA). Half maximal inhibitory concentrations (IC_{50}) were determined with GraphPad Prism 5.0a (GraphPad Software Inc., 2007, CA, USA).

Results and Discussion

Phagocytic capacity and efficiency of hemocytes from *Mytilus edulis* during spawning period are 60% lower than those from mussels after the spawning period (Figure 1). This observation is consistent with previous studies.[14,18,19] Li *et al.* suggest a trade-off of the energy to the spawning process to explain the effects observed since glycogen and protein levels in the mantle of *Pacific oysters* also decreased during this period.[14] As suggested by Cartier *et al.,* it is possible that the increased level of sex steroid hormones during spawning period plays a suppressive role on the immune system.[19,21,22] This possibility was verified in the common carp, where 17β-estradiol, progesterone and 11-ketotestosterone were shown to inhibit phagocytosis.[20] More studies should be done to explain how the immune system of *Mytilus edulis* could be affected by spawning. Since sex can also influence the immune and endocrine systems of bivalves,[23,24] it should be included in such studies.

Fourteen days after spawning, phagocytic capacity and efficiency of hemocytes return to the baseline levels founds before spawning period (Figure 1). In the pacific oysters, a

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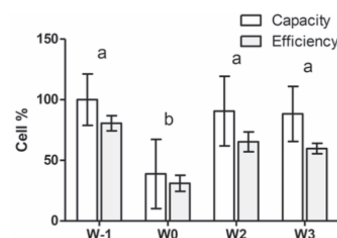

Figure 1. Phagocytic capacity and efficiency of hemocytes from Mytilus edulis according to spawning period. Mussels were sacrificed one week before (W-1) spawning, during (W0) spawning, two and three weeks after spawning period (W2 and W3, respectively).Different letters indicate, for capacity and efficiency, a significant difference (ANOVA, P<0.0001).

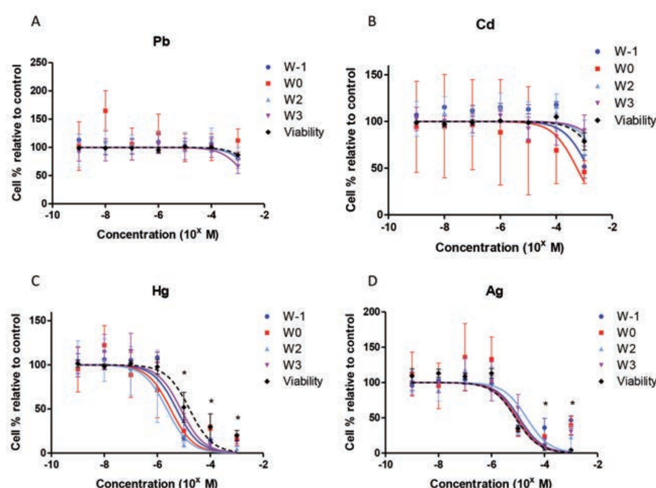

Figure 2. Phagocytic capacity and viability of hemocytes exposed to metals [A: lead (Pb), B: cadmium (Cd), C: mercury (Hg) and D: silver (Ag)] from Mytilus edulis in or out spawning period. Mussels were sacrificed one week before (W-1) spawning, during (W0) spawning, two and three weeks after spawning period (W2 and W3, respectively). Controls were not exposed to metals. To compare difference between concentrations: *ANOVA/Kruskal-Wallis P<0.05.

Table 1. Inhibitory concentrations (IC50) of phagocytic capacity of hemocytes exposed to metals in or out spawning period (expressed in 10^x M).

	Pb	Cd	Hg	Ag
W-1	> -3	> -3	-5.±0.2	-5.1±0.2
W0	> -3	-3.±0.7	-5.5±0.3	-5.0±0.5
W2	> -3	> -3	-5.6±0.1	-5.0±0.2
W3	> -3	> -3	-5.1±0.2	-4.7±0.3
Viability	> -3	> -3	-4.8±0.2	-5.1±0.1

Pb, lead; Cd, cadmium; Hg, mercury; Ag, silver. W-1, one week before spawning; W0, during spawning; W2, two weeks after spawning; W3, three weeks after spawning.

recovery is observed 8 days after the spawning.[14] Future experiment should include more time points to better understand the recovery of immune system of *Mytilus edulis* after spawning.

A significant decrease in hemocytes viability exposed to Ag and Hg at 10^{-5} M was observed independently of the spawning period (Figure 2C and D). A decreased in phagocytic capacity was associated.

Spawning did not affect phagocytic capacity following an exposure to Ag, Hg and Pb (Figure 2 A, C, D and Table 1). However, spawning decreased the phagocytic capacity of mussel hemocytes exposed to Cd (Figure 2B and Table 1). Thus, mussels in spawning period exposed to Cd could be more vulnerable to diseases than those not exposed to Cd, this remains to be examined further.[14-16,18,19]

Since spawning period was determined by the observation of gametes in the water, it is possible that some mussels sacrificed were still not in spawning process. Future experiments should include histological analysis of the gonads in order to determine the gametogenesis stage and the sex of each mussel. Nevertheless, this preliminary pilot study demonstrates the importance of considering the stage of reproduction of *Mytilus edulis* in studies of the immune system.

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