Fitoestrógenos Nutricionales como Inductores de Fenotipo Epigenético en Etapas Tempranas del Desarrollo en Mamíferos

Tesis

Entregada a la

Universidad de Chile

en cumplimiento parcial de los requisitos

para optar al grado de

Doctor en Ciencias con mención en Ecología y Biología Evolutiva

Facultad de Ciencias

por

Carlos M. Guerrero-Bosagna

Marzo, 2006

Directores de Tesis:

Dr. Pablo Sabat

Dr. Luis Valladares

FACULTAD DE CIENCIAS

UNIVERSIDAD DE CHILE

INFORME DE APROBACIÓN

TESIS DE DOCTORADO

Se informa a la escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por el candidato

CARLOS MARCELO GUERRERO BOSAGNA

Ha sido aprobada por la Comisión de Evaluación de la tesis como requisito para optar al grado de Doctor en Ciencias con mención en Ecología y Biología Evolutiva, en el examen de Defensa de Tesis rendido el día 26 de enero de 2006.

Director de Tesis:

Dr. A. Pablo Sabat .....

Dr. Luis Valladares .....

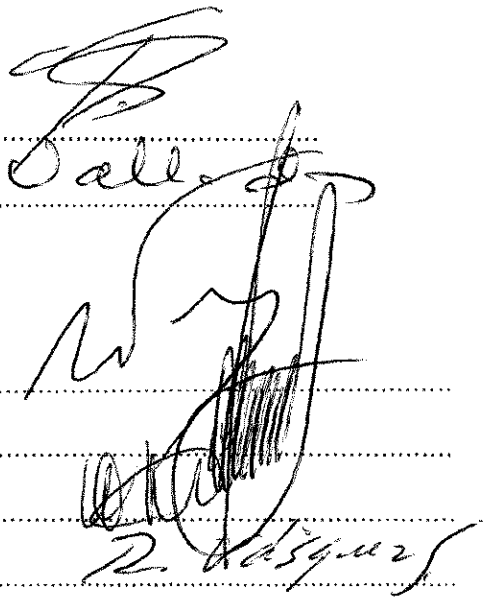
Comisión de Evaluación de la Tesis:

Dr. Alberto Veloso .....

Dr. Raul Fernandez .....

Dr. Walter Sierralta .....

Dr. Rodrigo A. Vásquez .....



A mi familia,

ao meu amor y a mis amigos

La forma no se explica, se hace

Roberto Matta

Chance is only the measure of our ignorance

Jules Henri Poincaré

Your mind should ever be on Ithaca.
Your reaching there is your prime goal,
But do not rush your journey anywise.
Better that it should last for many years,
and that, now old, you moor at Ithaca at last,
a man enriched by all you gained upon the way,
and not expecting Ithaca to give you further wealth.
For Ithaca has given you the lovely trip.
Without her you would not have set your course.
There is no more that she can give.
If Ithaca seems then too lean, you have not been deceived.
As wise as you are now become, of such experience,
You will have understood what Ithaca stands for.

Constantine P. Cavafy

AGRADECIMIENTOS

En primer lugar, quiero agradecer a aquellos que conforman la influencia más importante en nuestro desarrollo ontogenético cultural, la familia. Quiero agradecer a mis hermanos por ser cómplices y amigos en las ocasiones más importantes de la vida, a mi abuelita (Mamy) por toda su dedicación y entrega incondicional en educarnos y atendernos. A mi abuelita Adriana por su expresiva y entusiasta alegría incambiable al vernos. A mi Papá, por no dejar faltarme nada en esta vida, por todo su amor acogedor y por habernos educado sanamente y cercano al deporte, a la ciencia y a la biología. A mi Mamá, por ser la más incondicional de todas, la de la palabra de apoyo, de la sensibilidad inexplicable y de la comprensión infinita. A Renée, por todo el amor que nos une, por todo lo lindo que ha sido lo nuestro, por todo el apoyo incluso a distancia y por embarcarse junto a mí en el hermoso desafío de una nueva etapa de mi vida en Ithaca.

A mis tutores, apoyadores cada uno en su estilo, uno mas paternal y el otro mas exigente. Ello fue una combinación extraordinaria para el desarrollo de toda esta tesis. Debo agradecer a ambos también por haber creído en mis ideas cuando nada hacia factible desarrollarlas y por haberme dado la libertad de imaginar y desarrollar mi tesis, sin dejar de lado el consejo experimentado.

A los miembros de la comisión, quienes han sido todos entusiastas y aportadores en esta aventura de una tesis con tanto énfasis molecular en un programa volteado a la ecología.

Invaluable también ha sido el aporte y enseñanza de aquellos que no siendo tutores o parte de la comisión me entregaron medios y conocimientos indispensables para la realización de las distintas etapas de la Tesis. A Susan Clark, quien definitivamente se portó como una tutora más dentro de esta tesis, aportándome conocimiento, amistad, los medios necesarios y un ambiente extraordinario para el desarrollo tanto de experimentos, como de relaciones personales. A Carezza Botto, que me proporcionó sin condiciones medios indispensables para el desarrollo de esta tesis. A Pablo Lois, Roxana Pey y Soledad Fernández por enseñarme la difícil tarea de extraer blastocistos. A Fernando

Garrido por haberme entregado su vasta experiencia en el HPLC y por su paciencia en cada evento de medición. Al Profesor Canals por sus enseñanzas y toda su paciencia en estos años de preguntas estadísticas. A Beck por su amistad y paciencia increíble ensañándome el "arte" del Bisulphite Sequencing. A Carmencha por haberme tantas veces cuidado los ratones. A Lorena Santa María por compartir conmigo las alegrías y dificultades del doctorado mientras estuvimos en el INTA y por que su ayuda fue fundamental para poder postular a la Beca de Termino de Tesis.

A mis amigos, que han hecho que el paso por esta facultad sea ameno, lleno de recuerdos imborrables y lazos duraderos. Gracias chiquillos, Chuchuca, Manino, El más H...., Hombre Pozque de Chile, Pelao, Laytte, Loaysa, Enrico (lejos, pero siempre presente), Varguis, Yurina, Loli, Malu, por su felicidad, cariño sincero y por su amistad, por las curaderas de consuelo y también por las de felicidad. A Bacter, Diablo, Alexaurio, Feña, Razeto, Leo y todos los otros Decenines, por lo mismo y además por las muy intelectualmente fructíferas discusiones de los viernes por la tarde.

A mis pololas que tuve durante el período del doctorado y que representaron un importante sustento emocional y apoyo en momentos fundamentales, muchas gracias Leslie y Roxanna.

A mis amigos de la época del colegio en Brasil que aun perduran, Helda, Tati, Cabral, John, Gobane, Marcelo, pero en particular al Cassio, quién ha sido una influencia muy importante en mi vida que me ha hecho darme cuenta en la adolescencia que las cosas pueden ser vistas desde un punto de vista alternativo a lo habitual.

A mis tíos de Sydney, Alicia y Julio, y además a Oliver, por haberme recibido en dos ocasiones en su casa cuando realicé las estadías al laboratorio de Sue Clark.

No puedo dejar de recordar y también agradecer a aquellos profesores que han sido una influencia importantísima en mi formación como científico, recibiéndome en sus laboratorios cuando yo era un estudiante de pregrado y entregándome amistosamente sus conocimientos. Quiero aquí agradecer al Dr. Manuel Contreras por haber sido el primero en hacerme parte de su laboratorio, por haberme

propiciado las primeras oportunidades para hacer ciencia y por haberme tantas veces corregido el portuñol. A la Dr. Ana Preller por haberme enseñado como ser riguroso en la experimentación científica, y como esto se refleja en buenos datos. Al Dr. Jorge Mpodozis por haberme abierto la mente en cuanto a lo convencionalmente enseñado en evolución, por las discusiones filosóficas y el posterior trabajo que me llevaron por primera vez a ser parte de una publicación científica.

A Sandra Hirsch por abrirme la oportunidad de seguir aplicando mis conocimientos sobre metilación en un proyecto Fondecyt y por tenerme paciencia mientras terminaba la tesis. Además agradezco muy sinceramente la amistad y simpatía de la gente del laboratorio de Nutrición Clínica del INTA.

A los miembros actuales y pasados, de todos los laboratorios donde he estado desarrollando esta tesis: a los ecofisiólogos, Sandra, Bruno, Claudio, Andrés, DF, Gricel, Paty, Karin, Canals, Maritza, Carezza y Carmencha; a los de Hormonas y Receptores, Jenny Concha y Muñoz, Profesora Argelia, Anita, Miguel, Ferna, Marcela Vasquez y Verónica Segura; a los epigenetics, Beck, Wendy, Wenja, Kate, Alison, Caroline, Clare, Cheryl, Jenny, Aaron, Patrick y Jordi.

También agradezco profundamente a todas las instancias que me entregaron apoyo económico durante el doctorado. A CONICYT por Beca de Mantención y Termino de Tesis, a INTA y Nestlé-Chile S.A. por haberme concedido en tres ocasiones la Becas Dr. Abraham Stekel de estímulo a jóvenes investigadores, a DID-Universidad de Chile por beca para financiamiento de tesis doctoral, a MeceSup por haberme concedido beca en dos ocasiones para estadías en el extranjero, y también a los proyectos Fondecyt 1010647 a Pablo Sabat y Fondecyt 1030309 a Luis Valladares.

Finalmente quisiera dedicar este trabajo a dos personas que fueron una muy linda influencia en mi vida y que ya no están. Uno es Gonzalo Farfán (Conejo), entusiasta amigo, organizador, crítico, científico, pensador y compañero de laboratorio. El otro es "El Profe Rosenmann", quien para mi orgullo integró la comisión, me dejó muchas enseñanzas imborrables y pese a que ya no está, su huella está plasmada en esta tesis. Gracias Conejo, gracias Profe.

INDICE DE MATERIAS

RESUMEN

La variación de caracteres entre individuos de una población es un tema central en la teoría darwiniana de la Selección Natural, aún cuando los procesos involucrados en el origen de estas variaciones han recibido menor atención. Desde una perspectiva evolutiva, el origen de una especialización al interior de un linaje puede ser una interrogante distinta a la de la mantención, una vez originada. En ese sentido, el desarrollo embrionario sería tendría una gran relevancia sobre el origen de la variación fenotípica viable y susceptible de ser seleccionada, en tanto la Selección Natural determinaría a continuación cuales de esas variantes pueden prosperar y mantenerse en la historia evolutiva. Por lo tanto, es posible centrarse en el estudio de los procesos que llevan al origen de la variabilidad fenotípica heredable sin la necesidad de conocer si las novedades que surjan estarán sujetas a posteriores procesos selectivos. No obstante, para estudiar el origen de las novedades morfológicas y las restricciones en el cambio fenotípico se requiere del conocimiento acerca de los procesos que dirigen el desarrollo. Cambios de base epigenética que ocurren durante el desarrollo han propiciado la aparición de grandes transformaciones morfológicas a lo largo de la historia evolutiva.

Existen evidencias indicando que xenobióticos ambientales pueden interferir en el desarrollo embriológico temprano en los animales, lo que tendría consecuencias en el estado adulto. Durante el proceso embrionario el microambiente en el cual el feto se desarrolla es susceptible a influencias por parte de la madre y por lo tanto vulnerable a variaciones en el estado hormonal de ésta. Dicho estado hormonal puede ser profundamente alterado por el medio, a través de compuestos con acción estrogénica (CAEs). Los fitoestrógenos son CAEs que se encuentran en compuestos vegetales. Algunos de ellos, como daidzeína y genisteína, han sido encontrados en alimentos comunes en la composición dietaria natural de roedores.

Se ha descrito en roedores, que cambios en el epigenotipo derivados de la exposición temprana a CAEs pueden ser transmitidos a las generaciones subsiguientes. Por ejemplo, se conoce que la exposición temprana (blastocistos en cultivo) a compuestos estrogénicos sintéticos como el bisfenol-A, puede repercutir en rasgos en el adulto, como el peso. Incluso, se ha demostrado que la exposición *in utero* al dietilestilbestrol (DES) puede producir una aumentada susceptibilidad a determinados tipos de tumores y mas aún, dicho aumento se pude transmitir transgeneracionalmente sin la necesidad de haber sido estimulada nuevamente por el DES. Dicha herencia podría deberse a la acción de los CAEs sobre la metilación del DNA. Si a estos hechos agregamos que altas cantidades de fitoestrógenos dietarios pueden producir una considerable actividad estrogénica, comparable a la producida por DES, y que la exposición temprana en la ontogénia a fitoestrógenos puede inducir a alteraciones morfológicas y en el patrón de metilaciones de proto-oncogenes, es factible inferir que en roedores, alteraciones en el estado hormonal de hembras preñadas producidas por el consumo de fitoestrógenos afectan:

i) las señales en el ambiente intrauterino donde se desarrolla el embrión, y en consecuencia ii) el proceso de reestablecimiento de patrones de metilación de genes en el embrión en desarrollo, lo que produce iii) alteraciones tanto en el fenotipo adulto resultante, como en ciertos caracteres de historia de vida de una población.

Basado en lo anterior, el presente estudio evaluó en *Mus musculus domesticus* si CAEs de origen natural (fitoestrógenos dietarios, en este caso) actuando a través de la madre, podrían producir modificaciones epigenéticas en etapas tempranas del desarrollo, verificando además sus consecuencias en parámetros de historia de vida. Para ello, los roedores fueron sometidos a una dieta alta en fitoestrógenos *ad libitum*, conteniendo 2% de un extracto de isoflavonas de soja (*Glycine max*), alimento natural que presenta la mayor concentración conocida de los fitoestrógenos genisteína y daidzeína.

Se evaluó si someter hembras de roedores preñadas al consumo de fitoestrógenos podría producir alteraciones en los patrones de metilación del ADN, específico en genes y general en el genoma, tanto en el embrión preimplantacional como en hígado y páncreas en adultos. Además, se estudió si el consumo de fitoestrógenos por parejas de roedores puede desencadenar diferencias morfométricas y en parámetros de la historia de vida en la descendencia. En *Mus musculus* existe evidencia de que estos rasgos se ven modificados cuando se someten roedores neonatos a tratamiento con fitoestrógenos. Sin embargo, no existen estudios que demuestren este efecto en descendientes de hembras preñadas consumiendo altas concentraciones de fitoestrógenos.

En este estudio, los roedores fueron sometidos a dos tratamientos, en los que se les suministró *ad limitum* una de las siguientes dietas: i) alimentación por comida alta en fitoestrogenos (Dieta ISF), compuesta por pellet para ratón con 2% de extracto comercial de isoflavonas, y ii) alimentación por comida baja en fitoestrogenos (Dieta Control), que consistió solamente en pellet para ratón. Se evaluó el efecto del consumo de una dieta alta en fitoestrógenos (2 % isoflavonas) por una población experimental de ratones, en tres ámbitos:

i) fisiología intrauterina en madres, para lo cual se desarrolló una técnica para la determinación por HPLC de catecolestrógenos intrauterinos, cuya secreción podría verse afectada debido a un alto consumo de fitoestrógenos.

ii) caracteres morfométricos y de historia de vida en la descendencia, para lo cual se evaluaron parámetros como peso, tamaño, distancia ano-genital y fecha de maduración sexual.

iii) metilación de genes en la descendencia (embriones y tejidos adultos) de madres sometidas a los tratamientos, para lo cual se midió metilación del ADN en los genes α-actina y Receptor de Estrógeno α en adultos y en LINE (secuencia repetida) en embriones.

En cuanto a resultados experimentales, en el ámbito ecológico-poblacional se observaron en las crías importantes variaciones en respuesta al consumo de altas cantidades de fitoestrógenos. Entre estas variaciones destacan la disminución en el peso en machos adultos (parámetro morfométrico) y el adelantamiento de la maduración sexual en hembras (parámetro de historia de vida). En el ámbito molecular se observaron cambios en la metilación de sitios CpG en las crías, en respuesta al tratamiento con altas cantidades de fitoestrógenos dietarios. Los cambios fueron observados en adultos para el gen α-actina en hígado y páncreas, y para el gen ERα en hígado. Además, se observó reducción en mutaciones en dos sitios CpG en la secuencia repetida LINE en blastocistos de madres tratadas con una dieta alta en fitoestrógenos.

Por lo tanto, agentes ambientales como los fitoestrógenos, actuando en etapas claves de la ontogenia pueden inducir alteraciones tan importantes como i) en parámetros epigenéticos como la metilación del DNA en genes específicos, ii) en parámetros de historia de vida que pueden repercutir profundamente en la estructura de una población, como la edad de maduración sexual, y iii) en parámetros morfométricos como el peso animal adulto, lo que podría tratarse de un efecto epigenético asociado a genes parentalmente marcados (GPM) o solamente a un efecto fisiológico relacionado con la conversión de hormonas o la suplantación del estimulo hormonal por fitoestrógenos.

Estos resultados resaltan la importancia de dar mayor énfasis a los procesos evolutivos inductivos además de los procesos evolutivos restrictivos. Dentro de este enfoque, se enfatiza como los cambios evolutivos pueden surgir como respuestas organísmicas estructurales a estímulos ambientales específicos, que a su vez pueden actuar en ventanas temporales bien delimitadas durante la ontogenia, permitiendo dicha respuesta. Sin embargo, lo anterior no excluye la posibilidad de que estos cambios ambientalmente inducidos puedan transformarse a futuro en adaptaciones. Por otro lado, denota que el estudio de las adaptaciones y/o selección natural no es un tópico estrictamente

necesario para el estudio de la evolución. Es innegable que la forma en como el ambiente *restringe* la estructura de un organismo juega un rol importante en la formación de este. Sin embargo, no menos importante es que la formación del organismo también ocurre por como el ambiente *induce* cambios en dicha estructura, mecanismo que debe tomarse más en consideración en estudios evolutivos.

ABSTRACT

Variation in characters among individuals in a population is a central topic in the Darwinian theory of Natural Selection. Even more when the processes involved on the origin of such variations have received minor attention. From an evolutionary perspective, the origin of a specialization in a lineage may be treated as a different issue than of the maintenance of such specialization, once it is originated. In this sense, embryonic development would be the main responsible for the origin of the viable phenotypic variation and furthermore, capable to be selected. On the other hand, Natural Selection would then define which variant can prosper and perpetuate in evolution. Thus, it is possible to focus on the study of those processes leading to the origin of the heritable phenotypic variability without the need for knowing whether the novelties that eventually arise will be subjected to further selective processes. Nevertheless, to study the origin of the morphologic novelties and the restrictions on the phenotypic change it is required to know about the processes driving development. Epigenetic changes occurring during development have lead to significant morphological transformations along the evolutionary history.

There are some evidences showing that environmental xenobiotics can interfere during early development, which could have consequences on the adult stage. During the embryonic process, the microenvironment where the fetus develops is susceptible to influences from the mother and therefore, also susceptible to changes in maternal hormonal state. Such hormonal state can be strongly influenced by the environment, through the action of compounds with estrogenic effects. Some of those compounds can be found in vegetables. These are called phytoestrogens. Some phytoestrogens as daidzein and genistein can be naturally available for rodent consumption. It has been described in rodents that changes in the epigenotype produced after early exposure to compounds with estrogenic action (CEA) can be transmitted to the subsequent generations. Such kind of heredity could be attributed to the action of such CEA on DNA methylation. In addition,

there are evidences reporting that high amounts of dietary phytoestrogens are able to produce considerable estrogenic activity, comparable to that produced by DES, and that early ontogenetical exposure to phytoestrogens can trigger morphologic alterations and on methylation patterns on proto-oncogenes. Based on that evidences, I proposed that in rodents, alterations in the hormonal state of pregnant females produced by the consumption of phytoestrogens (or other xenoestrogens) affect: i) the hormonal signaling in the intrauterine environment where embryo development takes place, which in turn have consequences on ii) the process of resetting of methylation patterns in the developing embryo, which produces alterations in both the resulting adult phenotype and in life-history characters of a population.

The present study evaluated in *Mus musculus domesticus* if natural CAEs (in the present case, dietary phytoestrogens), acting through the mother could be able to produce epigenetic modifications at early stages during development, verifying also the consequences on life-history parameters. The experimental approach to investigate it consisted of feeding adult rodents *ad libitum* with a diet high in phytoestrogens, containing 2% of a soy (Glycine max) isoflavones-extract. Soy is the food item presenting the higher known concentrations of the phytoestrogens genistein and daidzein. It was evaluated if such high maternal consumption of phytoestrogens could be producing changes in DNA methylation patterns in two specific genes in the adult offspring, in liver and pancreas, or in general methylation along the genome in blastocysts. Morphometric and life history characters were also evaluated in the offspring of mice couples subjected to control diet or the diet containing 2% isoflavone-extract. Previous evidence in *Mus musculus* shows that these traits are modified when subjecting neonatal rodents to treatment with phytoestrogens. Nevertheless, no studies have reported these effects on offspring from pregnant mothers feeding high amounts of phytoestrogens.

In the present study, rodents were assigned to one of the following experimental treatments: mice were fed *ad libitum* on i) control diet (chow diet for rodents), or on ii) control diet plus 2% soy-

isoflavones concentrate (ISF diet). The effect of the consumption of a diet high on phytoestrogens was assessed in an experimental population of mice. Three levels of analysis were defined to evaluate the hypothesis. The experimental approach used in this thesis focused on:

i) the physiological level on mothers, developing a technique capable of detecting and quantifying intrauterine catecholestrogens through HPLC, since secretion of these compounds could be affected by a high consumption of phytoestrogens;

ii) the molecular level, measuring DNA methylation in the offspring (blastocysts and adults) of mothers consuming high amounts of phytoestrogens, on genes α-Actin and Estrogen Receptor-α in adults and LINE repeated sequence in blastocysts;

iii) the ecological level in the adult offspring, measuring morphometric and life-history characters, such as weight, size, ano-genital distance and timing of sexual maturation, in the offspring of mice couples subjected to control or ISF diet.

With regard to the experimental results, in the ecological level important changes were detected in the offspring in response to the phytoestrogenic treatment, among them, reduction in size of adult males (morphometric parameter) and advancement of sexual maturation in females (life-history parameter). At the molecular level, changes in methylation in CpG sites were observed in the offspring in response to the phytoestrogenic treatment. Changes were detected in adults for α-Actin gene in liver and páncreas, and for ERα gene in liver. In addition, reduced mutations were observed in two CpG sites in LINE repeated sequence in blastocysts from mothers consuming high quantities of phytoestrogens.

Thus, environmental agents as phytoestrogens, acting in key stages during ontogeny are able to induce important alterations as: i) on epigenetic parameters as gene specific DNA methylation, ii) on life-history characters that influences population structure, as timing of sexual maturation, and iii) on morphometric parameters such as adult weight, which may represent either an epigenetic effect

associated to Imprinted Genes, or to a physiological effect related to mimicking of hormonal stimuli by phytoestrogens.

These results highlight the importance of giving more emphasis to inductive evolutionary processes in addition to restrictive evolutionary processes. This focus emphasises how evolutionary changes may arise as structural organismic responses to specific environmental stimuli, which can in turn act in very limited windows of time during ontogeny, making possible such a response. Nevertheless, the former do not exclude the possibility that such environmentally induced changes may be further transformed in adaptations. In parallel, such focus reinforce that the study of adaptations and/or natural selection is not a requirement for studying evolution. It is undeniable that the way in which the environment *restricts* the organism structure is fundamental for the shaping of it. Nevertheless, as important is how the environment *induces* changes that help to shape the organism. Such a mechanism should be more considered in studies of evolutionary biology.

I

INTRODUCTION -

This thesis is conceived on the basis of my interests on the phenomena of epigenetics, on the role of environment on inducing evolutionary changes, and on the early embryo as an important stage in ontogeny, as a target for environmental effects and also as a source for producing evolutionary changes. Environmental estrogens were a primary source of interest, given the incipient evidences on possible epigenetic effects on animals, especially on the best known epigenetic mechanism to date, DNA methylation. Besides, their naturally availability for consumption by a variety of animals lead me to think of phytoestrogens as a natural source candidate for inducing evolutionary changes.

An extensive review on the matter and speculations on the possible evolutionary role of environmental estrogens gave rise to a perspective article, which was published in Evolution and Development (vol. 7, N° 4, pg 341-350; 2005) with the name of "*Environmental Signaling and Evolutionary Change: can exposure of pregnant mammals to environmental estrogens lead to epigenetically induced evolutionary changes in embryos?*". The paper exposes all the theoretical support and evidences for the hypothesis proposed in this thesis, and is included as the Part II.

Briefly, the evidence considered for the construction of the hypothesis is that, in rodents: i) a mother mediated early exposure to synthetic estrogenic compounds, can produce developmental alterations, sometimes with transgenerational consequences; ii) a naturally reachable high consumption of dietary phytoestrogens can produce a strong estrogenic activity, and iii) the early ontogenetical exposure to phytoestrogens can induce alterations both in morphological parameters and in methylation patterns in specific genes.

Based on that evidence, I proposed the following hypothesis:

In rodents, alterations in the hormonal state of pregnant females produced by the consumption of phytoestrogens (or other xenoestrogens) affect: i) the hormonal signalling in the intrauterine environment where embryo development takes place, which in turn have consequences on ii) the process of resetting of methylation patterns in the developing embryo, which produces alterations in both the resulting adult phenotype and in life-history characters of a population.

Considering the theoretical framework exposed in the abovementioned paper, three levels of analysis were defined to evaluate the hypothesis. The experimental approach used in this thesis focused on i) the physiological level on mothers, ii) the molecular level (DNA methylation) in the offspring (blastocysts and adults), and iii) the ecological level in the adult offspring. A summary of the work performed in each level is described next.

As previously mentioned, there is strong evidence suggesting that dietary consumption of high amounts of phytoestrogens by pregnant mice females could lead to variations in the secretions of the catecholestrogens 4-OH-Estradiol and 2-OH-Estradiol, which could in turn affect the process of DNA methylation in embryos. Nevertheless, no studies have developed a technique to measure the secretions of such compounds directly in biological secretions, which would be in turn useful to detect such catecholestrogens in the intrauterine secretions as well. This will permit to evaluate how intrauterine secretions of catechoestrogens are responding to high levels of maternal consumption of phytoestrogens. Therefore, the first goal of the present thesis was to develop a technique capable to detect and quantify both 4-OH-Estradiol and 2-OH-Estradiol in biological

fluids. These results gave rise to a manuscript, which is shown in the Appendix and named "*Separation and quantification of the catecholestrogens 4-OH-Estradiol and 2-OH-Estradiol by HPLC: an improved procedure for measurement of potential cancer biomarkers*".

The second goal of this thesis is exposed in the Part III, and is related with ecological effects of a high consumption of phytoestrogens in an experimental population of mice. Morphometric and life-history characters were evaluated in the offspring of mice couples subjected to control diet or a diet containing 2% of an isoflavone-extract. These results are included in the manuscript named "*Maternal Effects of Dietary Consumption of Phytoestrogens on Life-History and Morphometric Characters in Mice Offspring*".

The third goal of the present study was to evaluate if such high maternal consumption of phytoestrogens could lead to produce changes in gene specific or general DNA methylation patterns of the offspring. With that aim, we analysed in mice the consequences of the maternal consumption the 2% isoflavones diet on methylation profiles in the offspring. We analysed the promoter regions of 3 genes to check for effects on tissue specific gene methylation (skeletal α-Actin and ERα in liver and pancreas) and on global methylation changes (LINE elements). The results arising from these experiments are included in a manuscript "*Sex-specific methylation patterns change in offspring born to mothers consuming a diet rich in phytoestrogens*", which is shown in the Part IV of this thesis.

The Part V of this thesis is devoted to expose general conclusions of the present study and also to point out the new questions that emerged from the experimental work performed.

II

EVOLUTION & DEVELOPMENT 7:4, 341–350 (2005)

Environmental signaling and evolutionary change: can exposure of pregnant mammals to environmental estrogens lead to epigenetically induced evolutionary changes in embryos?

Carlos Guerrero-Bosagna,[a,b,*] Pablo Sabat,[b,c] and Luis Valladares[a]

[a]Laboratorio de Hormonas y Receptores, Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, Santiago, Chile

[b]Laboratorio de Ecofisiología Animal, Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago, Chile

[c]Center for Advanced Studies in Ecology & Biodiversity and Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile

*Author for correspondence: (email: catelo@uec.inta.uchile.cl)

SUMMARY DNA methylation is one of the epigenetic and hereditary mechanisms regulating genetic expression in mammalian cells. In this review, we propose how certain natural agents, through their dietary consumption, could induce changes in physiological aspects in mammalian mothers, leading to alterations in DNA methylation patterns of the developing fetus and to the emergence of new phenotypes and evolutionary change. Nevertheless, we hypothesize that this process would require (i) certain key periods in the ontogeny of the organism where the environmental stimuli could produce effects, (ii) particular environmental agents as such stimuli, and (iii) that a genomic persistent change be consequently produced in a population. Depending on the persistence of the environmental stimuli and on whether the affected genes are imprinted genes, induced changes in DNA methylation patterns could become persistent. Moreover, some fragments could be more frequently methylated than others over several generations, leading to biased base change and evolutionary consequences.

INTRODUCTION

An old question in evolutionary biology is "how does variation originate?" No matter how old this question is, the controversy remains regarding whether (i) variability in populations appears exclusively by random mutations, a position defended by neo-Darwinism, or (ii) the formation of novel characters can, in some way, be induced by external environmental forces. Current understanding of epigenetic modification of DNA shows that such controversy still exists. In this sense, Jaenisch and Bird (2003) suggested that future lines of investigation should place emphasis on the identification of the stimuli that can initiate evolutionary changes. They proposed that it is possible for external factors, such as dietary compounds, to lead to the accumulation of epigenetic changes over the years within populations. Given the recent evidences on mechanisms of epigenesis, here we propose that under certain conditions, such epigenetic changes could become persistent over generations and this could have evolutionary genetic consequences in a lineage.

We believe that a first approach toward evaluating this problem requires separating the phenomenon of the emergence of an evolutionary novelty into two processes: (i) that responsible for the origin of a new character and (ii) that maintaining such a character over generations (i.e., fixation). Such separation has been previously proposed by authors such as Futuyma and Moreno (1988) and West-Eberhard (1998).

As Darwin, most evolutionary biologists have concentrated almost exclusively on the second process, that is the form in which an evolutionary novelty can be fixed, not inquiring into the problem of how evolutionary novelties originate. Variation among individuals and correlated differences in fitness became a central topic in Darwin's theory (Endler 1986) and thereafter, Neo-Darwinian theory interpreted changes in allelic frequencies of populations instead of studying the origin of new phenotypes (Nijhout et al. 1986).

In accordance with the separation between origin and fixation of an evolutionary novelty, some authors state that evolution is always a two-step process, first involving

developmentally mediated variation, and then selection, whose operation results in gene frequency changes (Wake and Larson 1987; West-Eberhard 1998). In this sense, changes arising because of alterations in early developmental processes, which, furthermore, could, in some cases, be environmentally induced, can appear whether or not such changes could become fixed and prosper in a population. Hence, in our opinion, the diversity and evolution of species should be explained not only by those selective processes imposed by the environment but also by the action of the environment as an inductor of genotypic and phenotypic variation, which is the material basis for selection.

Regarding the persistence of such epigenetic changes through generations, long ago, Weismann (1893) stated that external influences may produce hereditary variations when they are capable of modifying the determinants of the germ plasm. Nevertheless, this could be only one of the ways through which environmental factors induce transgenerational epigenetic changes. We recognize two ways for this to occur: one is by dramatically modifying DNA aspects in the germ line with transgenerational consequences, that is by means of producing mutations or transgenerationally persistent epigenetic modifications in the genome, and the other is through inducing ontogenetical variation at every generation, although not producing inheritance through the germ line. From our perspective, inductive environmental forces can act to create, through one or both of these forms, new conformation of organisms, which also implies new possibilities within its surrounding environment. Jablonka and Lamb (1995) have named the range of the possible responses of individuals to new environmental challenges as the "reaction range" of individuals.

Based on his experiments in *Drosophila*, Waddington proposed two new concepts related to the capacity of environmental influences to induce the appearance of new characters in organisms and their maintenance over generations. First, in the face of disturbing and external stressing influences, there are counteracting tendencies in development toward normal adult conditions (i.e., canalization; Waddington 1959). Second, whereas these counteracting tendencies exist, if a stressing stimulus is capable of developmentally modifying a strain of organisms, the derived population may evolve exhibiting the modification even in the absence of the stress (Waddington 1952). He termed this process "genetic assimilation."

An important fact to notice is that, through these concepts, Waddington distinguished particular environmental stimuli capable of inducing epigenetic changes, which are the "stressing" ones. McClintock (1984) also stated that a particular kind of stimuli producing stress lead to a genome's reaction to it, whose response may underlie formation of new species. Furthermore, she stated that genome produces programmed responses, although it is necessary to subject the genome repeatedly to the same challenge in order to observe the nature of the induced changes.

At present, it is widely known that DNA methylation is one of the epigenetic and hereditary aspects that regulate genetic expression in mammalian cells (Khosla et al. 2001). Furthermore, DNA methylation is capable of being modified by the action of externally applied agents (Mac Phee 1998). Not all, but particular compounds found in nature could act as such agents. Moreover, they could be capable of affecting the evolution of organisms, inducing profound changes in individuals and populations, perhaps with transgenerational consequences. We hypothesize that, whereas certain conditions are required for this process to occur, it is a feasible phenomenon. The task is to identify the conditions constraining such a process.

Experimental evidences concerning alterations of methylation patterns, at least in mammals, are generally restricted to studies of the effects of synthetic compounds or dietary restrictions of food items containing the methyl group (see Laird and Jaenish 1996; Singal and Ginder 1999). Although this is very important for understanding the mechanisms of DNA methylation, from an evolutionary perspective, it is of greater relevance to find compounds that are naturally in contact with organisms; for example, those available for dietary consumption, which, in addition, could produce alterations in patterns of DNA methylation in organisms.

In this article, focusing exclusively on the phenomenon of how evolutionary novelties originate, we describe how in mammals, certain natural agents could induce alterations in particular mechanisms of regulation of gene expression in individuals, such as methylation patterns, and the further arising of new, specific phenotypes in subsequent generations, leading to evolutionary change. Nevertheless, we hypothesize that this process would require (i) certain key periods in the ontogeny of the organism where the environmental stimuli could produce effects, (ii) particular environmental agents as such stimuli, moreover, acting persistently, and (iii) that a persistent genomic change be consequently produced in a population.

The first requirement emerges because not all compounds are capable of producing an effect on mothers that will have consequences on the fetus; the second emerges from the fact that an organism is not equally sensitive to outer stimuli throughout ontogeny; and the third because transgenerational persistency of characters is ensured when it reaches the genomic level. Each of the three requirements presented will be more extensively treated later in the text.

DNA METHYLATION: EPIGENETIC IMPRINTING ON THE GENOME

Experimentation on the problem of how evolutionary novelties arise and the consequences on the genetic system of exposition to an environmental stimulus have been the

focus of epigenetic studies in a variety of organisms, including *Drosophila* (Rutherford and Lindquist 1998), bacteria (Cairns et al. 1988), and yeast (Steele and Jinks-Robertson 1992).

Several types of epigenetic inheritance have been described to date. Jablonka and Lamb (1995) have proposed three systems of epigenetic inheritance: (i) *steady-state systems*, such as Wright's (1945) persistence of alternative cellular states as a result of changes in nuclear genes or in cytoplasmic constituents of the cell, (ii) *structural inheritance systems*, such as the maintenance through generations of the ciliary patterns in protozoa, albeit of the genetic constitution of the cells involved (Nanney 1985), and (iii) *chromatin-marking systems*, or those related to the transmission of specific patterns of the chromatin structure (Holliday 1987; Jablonka et al. 1987). Specifically, the latter refers to non-DNA parts of the chromosomes that are capable of binding proteins or additional chemical groups attached to DNA bases, which affect the nature and stability of gene expression, now commonly named genomic imprinting. DNA methylation describes a postreplicative modification, in which a methyl group is added to a DNA residue in a covalent manner (Laird and Jaenish 1996); for this reason, it is a form of genomic imprinting. The DNA methylation reaction is enzymatically catalyzed by DNA methyltransferases (Dnmts) and takes place in 5′ to 3′-oriented CG dinucleotides, which are known as CpG sites, at the carbon 5 of the cytosine ring (Singal and Ginder 1999). CpG islands are regions with a high frequency of CpG sites; these islands are often associated with genes, and are usually found in promoter zones (Gardiner-Garden and Frommer 1987). CpG sites are not evenly distributed within the genome, and are preferentially unmethylated, regardless of the transcriptional activity of the associated gene (Bird 1986). As other regions are normally methylated, patterns of genomic DNA methylation can be distinguished along the genome (Singal and Ginder 1999; Bestor 2000; Jones and Takai 2001). Nevertheless, there is controversial information regarding whether methylation patterns are established because of the enzymatic activity of one or more Dnmts (Bestor 2000; Yokochi and Robertson 2002).

There are at least three families of Dnmts described to date: Dnmt1, Dnmt2, and Dnmt3. However, there is no agreement regarding whether each one plays a specific, differential role in the process of DNA methylation (Bestor 2000). It has been speculated that Dnmt3A and Dnmt3B are responsible for the establishment of methylation patterns during early development, whereas Dnmt1 is responsible for the further maintenance of such patterns. Experiments conducted in vitro support this model, revealing that Dnmt1 has a preference for hemimethylated DNA as a substrate (Yoder et al. 1997), whereas Dnmt3A and Dnmt3B act as a de novo methyltranferase, preferring unmethylated DNA (Yokochi and Robertson 2002).

There are multiple isoforms of Dnmts, but all are encoded by the same cytosine–Dnmt gene (Deng and Szyf 1998). Among these isoforms, Dnmt1o is a variant of Dnmt1 that accumulates in oocyte nuclei during the follicular growth phase, and Dnmt3L is an isoform of Dnmt3a and Dnmt3b, but that lacks Dnmt enzymatic activity and interacts with Dnmt2a and Dnmt3b (Kierzenbaum 2002). Dnmt3L acts as a cofactor for *de novo* methylation of imprinted genes in the female gametes and for the establishment of methylation imprints in oocytes (Hata et al. 2002).

It is worth noting that Dnmt1 is localized principally in somatic cell nuclei, but it is cytoplasmatic in the oocyte and in the preimplantation embryo (Bestor 2000). However, the variant Dnmt1o has transient nuclear localization in the eight-cell stage, corresponding to the time when genomic imprints are established (Howell et al. 2001). On the other hand, Dnmt3L co-localizes with Dnmt3a and Dnmt3b in mammalian cell nuclei (Hata et al. 2002).

Given the crucial role of the diverse Dnmts in the epigenetic modification of DNA, it is of great interest to know whether there are environmental substances capable of modifying the intracellular levels of such enzymes or their patterns of gene expression. Nevertheless, no studies have reported this kind of interaction, which we suspect may have a role in relating environmental stimuli to DNA modification. However, the recent finding that individual Dnmts can be tracked, and that their binding to genomic DNA can be quantified in vivo in mammalian cells (Liu et al. 2003) can be enormously helpful for determining the link between environmental compounds and the process of DNA methylation.

IMPRINTED GENES: DNA METHYLATION AND PERSISTENCE OF MARKS THROUGH GENERATIONS

Roemer et al. (1997) were the first to show reappearance in the progeny of modified characters in parents. In their experiments on rodents, the adult phenotype produced because of the fusion of pronuclei with eggs of different genotypes was also observed in the offspring. Furthermore, such transgenerational persistence of the modified characters was related to altered methylation patterns that were, in turn, transmitted through male gametogenesis. However, not all genes are equally capable of passing on changes in patterns of methylation. There is a particular class of genes, crucial for understanding the mechanisms of epigenetic inheritance, that are known to have relatively unchanged methylation patterns over generations. These genes, named "imprinted genes", do not seem to be affected by overall alterations in methylation patterns that take place early in development (Constância et al. 1998). Such genes carry a molecular memory of their parental origin that is acquired early in the germ line (Surani

2001). This molecular memory is associated with specific methylation patterns in CpG islands of each allele, which consequently affect further genic expression (Costello and Plass 2001).

Once the allelic differences in methylation of imprinted genes are defined (during the establishment of germinal line in the developing embryo), such differences generally remain stable in the somatic tissues (Constância et al. 1998). The marking process of these genes appears to involve three stages: (i) the establishment of marks in gametes; (ii) the permanence of these marks during embryogenesis and in the adult somatic tissues; and (iii) the erasure of marks in the early germ line (Razin and Cedar 1994). Conclusive information on the way in which methylation in imprinted genes is initiated from an unmethylated state during gametogenesis is still elusive (Ferguson-Smith and Surani 2001). However, recent investigations indicate that primordial germ cells are substantially methylated (which corresponds to the same pattern in somatic cells) before they colonize gonads and become demethylated around the time of entry into the gonads (Hajkova 2002). An incomplete deletion of marks during gametogenesis would explain the inheritance of the parental epigenotype (Reik et al. 2001).

CHANGING DNA METHYLATION AND ITS IMPLICATIONS

Imprinted genes may be susceptible to undergoing changes in methylation patterns during preimplantational development (Khosla et al. 2001). As imprinted genes tend to conserve methylation patterns from one generation to the next, changing methylation patterns in these genes could lead to the appearance of the derived alterations in the future generations. Therefore, if external agents are capable of inducing particular changes in methylation patterns in these genes, such changes could flourish transgenerationally. Moreover, this could take place in the absence of the stimuli that initially changed its methylation pattern, generating a process that would be a kind of Waddington's "genetic assimilation" but in imprinted genes.

Changes of methylation patterns in certain imprinted genes can generate associated specific phenotypes (see Morison et al. 2001 for examples). Particularly interesting, from our perspective, is the Beckwith–Wiedemann syndrome. Researchers suspect that this syndrome is related to the loss of imprinting in *Igf2*, and is characterized by somatic overgrowth, macroglossia, abdominal wall defects, visceromegaly, and an increased susceptibility to childhood tumors (Caspary et al. 1999). Therefore, in this case, a change in methylation patterns in a single gene can lead to phenotypic changes in several characters.

However, even if no one imprinted gene is affected when altered by an environmental signal, environmentally induced changes in methylation patterns could also become persistent if such changes, and the environmental conditions allowing the establishment of such changes, are both conserved throughout generations. This could occur whenever there is a concordance, an association between the environmental stimuli, the established DNA methylation patterns, and the resulting phenotype of an organism. For instance, if some natural agent can induce the loss of methylation in genes and produce phenotypic alterations (e.g., those modifications emerging from the loss of methylation in *Igf2*), a standard phenotypic pattern will arise every time the specific environmental stimuli lead to the establishment of particular patterns of methylation. Still, it is important to consider that this could be a broader phenomenon, and environmentally induced changes in methylation patterns could affect several other imprinted genes as well. As a result, an environmental stimulus would bias the phenotypic change toward certain types of phenotypes.

Nevertheless, the consequences of altering DNA methylation toward specific persistent patterns could imply mutation in those specific segments of the genome. For instance, it is known that a methylated cytosine is half-way to the substitution of a cytosine for a thymidine. The completion of conversion requires only a hydrolytic deamination reaction (Singal and Ginder 1999). Therefore, if some methylated sites are frequently methylated over several generations, it is possible that an eventual base change from cytosine to thymidine will occur more frequently than any other substitution. In fact, CpG sites are hotspots for transitions from cytosine to thymidine, generated by a spontaneous deamination of 5-methyl cytosine to thymidine (Coulondre et al. 1978). The result would be, as mentioned by West-Eberhard (2003), that "evolved sensitivity to environmental influence during gene expression could influence susceptibility to certain kinds of structural change during evolution."

EARLY DEVELOPMENT: A KEY STAGE DURING ONTOGENY

The first condition for our statement on environmentally induced evolution is that the process must occur early in ontogeny, before or during the establishment of the germ line in metazoa. This is important for two main reasons: first, because eventual reprogramming of methylation patterns in the germ line can be transmitted to the progeny (Surani 2001), and second, because during development, there is an enhanced susceptibility of the organism to the action of outer compounds, with greater consequences in the adult than when the same stimulus occurs later in ontogeny (Amzallag 2000). With regard to the latter statement, Gould and Lewontin

(1979) have emphasized that during the early ontogenetic stages of complex organisms, "differentiation of organ systems and their integration into a functioning body is such a delicate process so easily derailed by early errors, with accumulating effects."

The morphogenic process of an organism is basically the product of a three-way interaction between the environment, genetic factors, and those characteristics that emerge from a self-organized dimension created by development itself (Amzallag 2000). The establishment of methylation patterns during early development (as well as other processes in morphogenesis) also depends on the immediate environment experienced by the embryo. These methylation patterns will guide the formation of particular cell types by controlling gene expression (Holliday 1998), therefore biasing further morphogenesis.

In mammals, patterns of methylation are established for the entire genome at least three times during development. The periods in which reprogramming of methylation patterns takes place are: (i) before the implantation of the embryo, (ii) during the development of the germ line (Reik et al. 2001), and (iii) during the period beginning soon after blastocyst implantation (Constância et al. 1998) until gastrulation (Mac Phee 1998). Before blastocyst implantation, a great part of the DNA is demethylated (Dean et al. 1998); thus, the DNA of blastocysts hardly shows methylation (Mac Phee 1998). Between blastocyst implantation and gastrulation, there is a wave of de novo methylations that restore the overall methylation patterns, which is retained in the somatic cells of animal for the rest of its life (Mac Phee 1998). In the germ line, reprogramming takes place by overall demethylations and methylations of the genome (Constância et al. 1998). In mice, primordial germ cells undergo an overall demethylation process in early development until day 13 or 14 (Reik et al. 2001). Later, during gametogenesis, there is a de novo methylation event until the previously observed high levels of methylation in the zygote (Mac Phee 1998), oocyte, and sperm genomes (Reik et al. 2001) are reached. It is likely that both demethylations taking place during the first stages of postzygotic cleavage, and methylations occurring after implantation, are important in removing acquired epigenetic modifications, especially those acquired during gametogenesis (Reik et al. 2001).

ROLE OF REPRODUCTION IN TRANSMITTING ENVIRONMENTALLY INDUCED ALTERATIONS IN METHYLATION PATTERNS

Reproduction involves the conservation in the progeny not only of the structure required to carry out the self-conserved organization represented by the organism but also the preservation of the structural characteristics of the environment that allow such organization to take place (Maturana-Romesín and Mpodozis 2000). An experimental approach to such a statement comes from Clark and Galef (1995), who proposed that daughters tend to resemble their mothers not only because both share a relatively large proportion of their genes but also because they tend to have similar histories of fetal exposure to steroids.

Applying this view to DNA methylation, reproduction plays a key role in passing on those changes in patterns of methylation that could eventually arise during early stages of the ontogeny. Reproduction, in addition to conserving the pattern of DNA methylation of an organism's genome throughout generations in a lineage, will also conserve the conditions allowing such patterns of methylation to be established in every generation. Hence, for a mammal to be formed from a zygote, and for development to take place generating a phenotype similar to the parental phenotypic pattern, the process requires not only the genetic content that provides a zygote with the potential to become an adult but also a surrounding environment for the embryo, which ensures the occurrence of appropriate methylations, at key periods of time during the embryological process.

Nevertheless, in mammals, despite the fact that the uterus acts as a buffer for either mechanical or chemical perturbations on the developing embryo, making the developmental process more isolated from environmental perturbations than in other taxa, the development is still susceptible to particular perturbations. Maternal effects such as variations in the hormonal status of a mother are capable of affecting the microenvironment in which the fetus develops (Clark and Galef 1998) and, consequently, its later ontogenetic processes (Bernardo 1996). For example, studies have shown that differential exposition to hormones can affect characters of the embryo. Clark et al. (1993) and Vandenbergh and Huggett (1994) demonstrated that the intrauterine position of female rodents affects the sex ratio of their litters, which is because of differential prenatal exposure to steroidal hormones, which in turn depends on the gender of neighboring embryos.

Besides, the hormonal state of the mammalian female can be strongly influenced by the environment through compounds that are naturally found in her diet (Nagao et al. 2001). In this sense, it has been reported that feed toxicants, or dietary imbalances of specific nutrients, can alter the composition of oviductal and uterine secretions (McEvoy et al. 2001).

Thus, the establishment of methylation patterns in the embryo is a process that depends directly on the environment in which it takes place, that is the intrauterine environment, but also indirectly on the surrounding environmental signaling, which, in some way, alters such an intrauterine environment. Accordingly, perturbing the intrauterine environment while early development takes place could bring about

consequences in the establishment of methylation patterns, with the corresponding phenotypic repercussions.

ENVIRONMENTAL AGENTS PRODUCING MOTHER TO FETUS INDIRECT EPIGENETIC EFFECTS

Our second condition is that only particular compounds in nature could act as environmental inputs for environmentally induced evolution to take place. The early embryo is exquisitely sensitive to alterations in its environment (McEvoy et al. 2001). Nevertheless, not every compound with which a mammalian mother has contact in nature is capable of altering the embryo environment, although some compounds could lead to alterations in mammalian hormonal features. Furthermore, we believe that some environmental compounds can, in addition to altering the hormonal status of a mammalian mother, be in turn capable of affecting important processes during the early development, including the establishment of methylation patterns in the embryo. Among those environmentally available compounds capable of affecting the hormonal status of a mammalian mother, there are some of synthetic origin, or xenobiotics (Danzo 1998) and of natural origin, such as phytoestrogens. The latter refers to secondary metabolites produced by plants (Croteau et al. 2000; Yu et al. 2000) that produce estrogenic action at a variety of levels in animals (McLachlan 2001). Phytoestrogens are readily available in the environment for animal consumption and their physiological, hormonal, and nonhormonal effects in animals have been studied to some extent (Levy et al. 1995; Santell et al. 1997; Boettger-Tong et al. 1998; Milligan et al. 1998; Gallo et al. 1999). Some phytoestrogens such as genistein and daidzein belong to a class of flavonoids, the so-called isoflavones (Liggins et al. 2000). The consumption of isoflavones can elicit uterotrophic and mammatrophic effects in mice and on the hypothalamic/pituitary axis as well (Santell et al. 1997). In humans, it has been reported that the consumption of phytoestrogens affects levels of the sex hormone-binding globulin, which regulates the bioavailability of steroidal sex hormones (Pino et al. 2000).

Changing the hormonal status in mammals could have consequences beyond the physiologic level. McLachlan (2001) suggested that estrogens could play a role in programming or imprinting those genes involved in cell proliferation, differentiation, or survival, either directly or through related signaling pathways. He also proposed that an estrogenic chemical may directly imprint a gene through a process leading to persistent genetic change, probably at the level of DNA methylation. In this sense, Barrett et al. (1981) suggested that diethylstilbestrol (DES), a powerful estrogenic synthetic compound, could transform cells by mechanisms other than punctual mutations, frameshift mutations, or small deletions. Currently, one could also interpret this cell transformation as alterations in methylation patterns. Some evidence for this phenomenon comes from studies in chicken liver, where estrogens appear to act in the regulation of expression of the vitellogenin I and II, and VLDL II genes, through changes in patterns of methylation of estrogen-responsive element sites (Edinger et al. 1997). It has also been shown that neonatal exposure to DES and adult ovary hormones produces abnormalities in the demethylation of the lactoferrine promoter, which shows that either hormonal xenobiotics or natural hormones are capable of triggering impairments during the development of organs (Li et al. 1997).

It has been reported that environmental estrogens can also produce direct effects on DNA methylation patterns. For example, administration of the phytoestrogens cumestrol and equal to newborn mice can enhance methylation and produce inactivation in the proto-oncogene *H-ras* (Lyn-Cook et al. 1995). In addition, Day et al. (2002) demonstrated that methylation patterns can be altered in 8-week-old mice that consumed high quantities of genistein.

With respect to hormonal effects early in development, Holliday (1998) was the first to envisage a possible link between hormone action and establishment of DNA methylation in mammalian embryos. He proposed that the effect of teratogens on a mother might disrupt the normal distribution of DNA methylation in a developing fetus, producing developmental abnormalities or defects that can appear in the subsequent generations. Newbold et al. (2000) reported that after administering DES to pregnant rats during early postimplantational development and neonatality, a greater susceptibility for specific tumor formation in rete testis and reproductive tract tissues occurred in F1 and appeared further in the non-DES exposed F2. These authors speculated that this transgenerational phenomenon could implicate epigenetic alterations that were transmitted through germ line, including changes in methylation patterns. Although this finding strongly suggests alteration and further transmission of a genomic change through germ line across more than one generation in response to an early exposition to an estrogenic compound, there is still missing evidence on the mechanism behind this process and whether it implies changes in DNA methylation patterns.

In the experiments of Newbold et al. (2000), the transgenerational persistence of the enhanced susceptibility to tumor formation takes place when mothers are exposed to DES after embryo implantation; however, estrogens play an important role even before implantation occurs. The implantation process involves complex interactions between the blastocyst and the uterus (Paria et al. 1993). Uterine preimplantational estrogen secretions are essential for activating the blastocyst of *Mus musculus* for further implantation, which is not possible if estrogen secretions are prevented by ovariectomization (Paria et al. 1998). Nevertheless, just as

estrogenic stimuli are needed for normal development, preimplantational exposure to synthetic estrogenic compounds can lead to phenotypic alterations. For instance, Takai et al. (2000) reported that in utero preimplantational exposure of rodent embryos to the synthetic estrogen bisphenol-A leads to an increased body mass of the animals at weaning. Furthermore, Wu et al. (2004) have recently shown that in vitro early exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin can indeed alter DNA methylation patterns in preimplantational embryos. Interestingly, those genes changed, *H19* and *IGF-2*, were imprinted genes.

Although it is not known whether compounds with estrogenic action (CEA) inside the uterus could act directly upon the developing embryo, or via intermediaries, it is possible that the relationship between estrogenic stimuli and methylation in the preimplantational embryo is mediated by the expression of *c-fos*. While on the one hand it is known that *c-fos* directly regulates the *dnmt1* transcription, increasing Dnmt1 levels (Bakin and Curran 1999), on the other, the induction of *c-fos* is a response attributed to membrane-mediated estrogen actions (Das et al. 2000). Through this mechanism, which provides an alternative pathway to the classical estrogen receptors α and β, CEA could trigger responses, as has been observed in pancreatic β cells (Nadal et al. 2000). In summary, the membrane-mediated estrogenic actions would first induce *c-fos* and then trigger the activation of the Dnmt1 enzyme.

Furthermore, in blastocysts, this indirect and membrane-mediated relationship between estrogenic stimuli and *c-fos* activation could also occur. In preimplantational blastocysts, Paria et al. (1998) demonstrated that latent blastocysts can be activated if they are incubated in vitro with 4-OH-17β-estradiol, a catecholestrogen synthesized from 17β-estradiol in uterine luminal epithelia by the action of the hydrogen-2/hidroxilase-4 enzyme. This response to 4-OH-17β-estradiol could also occur via a pathway distinct from the classical nuclear estrogen receptors (Paria et al. 1998). In addition, Paria et al. (1998) found that 4-OH-17β-estradiol increases with the epithelial growth factor (EGF) receptor. Interestingly, other studies have demonstrated that an increase in the EGF receptor may also be related to activation of *c-fos* (Kamiya et al. 1996). On the other hand, a direct induction of *c-fos* by estrogen has also been shown in different cell types (Allen et al. 1997; Garcia et al. 2000), which occurs via an estrogen receptor element present in this gene (Hyder et al. 1992). Thus, estrogenic stimuli could induce *c-fos*, either directly, through a gene receptor, or indirectly through membrane-mediated reactions.

Furthermore, phytoestrogens could also induce *c-fos* and consequently alter methylation patterns in cells. A study supporting this view demonstrated that the intake of genistein in ovariectomized female rodents induced the expression of the RNA messenger of *c-fos* in the uterus (Santell et al. 1997). Hence, we suspect that phytoestrogens can also act on blastocysts, which could occur through membrane-mediated estrogen actions, directly induced by isoflavones in uterine secretions, or mediated by other compounds secreted in the uterine epithelia such as 4-OH-17β-estradiol. The formation of this compound in uterine epithelia could be related to plasmatic isoflavone content, although no studies have attempted to detect such compounds in uterine secretions, or showed that its high consumption can alter the production of cathecolestrogens in the uterine epithelia.

Although there is strong evidence suggesting that the hormonal status of mammalian mothers can be an important feature related to the establishment of methylation patterns in early embryos, so far, there is no concluding evidence of this. We believe that an investigation on this subject should be performed in order to uncover the aspects behind an eventual epigenetic role of estrogenic compounds (both animal produced, plant produced, and synthetic) on developmental processes, in particular, on the establishment of methylation patterns in the early embryo.

THE "GENOMIC CHANGE" REQUIREMENT FOR A PROCESS TO BE CONSIDERED EVOLUTIONARY

The third requirement that we propose for the environmental and hormonal induction to become an evolutionary process is that genomic change should be achieved. Evolutionary change in the morphogenetic process must arise from changes in patterns of regulation and interaction during ontogeny (see discussion by Atchley 1987). Such a connection gains special importance when considering that the patterns of regulation and interaction occurring at early stages in ontogeny could, even in mammals, be susceptible to environmental changes.

Nevertheless, the question arising at this point goes beyond the relationship between the environmental stimuli and eventual epigenetic consequences on DNA methylation. The challenge is to know how an eventual change in DNA methylation patterns could become persistent and evolutionary. Besides, another question arises, regarding the definition of evolutionary change. Is persistence in the conditions allowing the establishment of changed methylation patterns across lineages a sufficient attribute for such changes to be considered as evolutionary, or do such changes need to reach the threshold of mutation at the genomic level?

It is true that genomic mutational change ensures a great degree of persistence through generations. However, persistence can also be the result of two processes, as previously mentioned: (i) the environment could persistently trigger, generation after generation, a specific change in methylation patterns, or (ii) persistence could be present in intrinsic features of the organisms as, for instance, the stable nature of the DNA.

Given the special feature of imprinted genes regarding possessing methylation patterns that are more stable across generations than other genes, persistence could be achieved through changing methylation patterns of imprinted genes. In this view, such changes in imprinted genes could have the same evolutionary value of mutations, given that there is an associated character variation with the changes, and because of the persistence of these changes throughout generations. Thus, the definition of "evolutionary change" at this point becomes blurred. What is true is that persistence through generations could be achieved in alternative ways to genomic mutation. Nevertheless, speaking in terms of genomic mutation, this could be achieved when the persistent change in methylated cytosines bias to specific mutations, as previously mentioned.

Regarding the frequency of eventual mutations derived from changes in methylation patterns, given that such changes can be environmentally induced, they cannot be considered to be at random. Therefore, we can expect that in these cases, the appearance of mutation will be in greater frequency than when mutation is considered to be at random. In fact, there is a 12-fold higher than normal mutation rate for the conversion of the methylated form of CpG to TpG and CpA, which reduces the occurrence of CpG to about 20% of its expected frequency in vertebrate genomes (Sved and Bird 1990).

Despite the evidence suggesting that the environment, through the action of naturally consumed agents, can alter the developmental process to the point that the emerging alterations can be inherited as evolutionary change, conclusive information is still elusive. Evidence in the direction of genomic change derived from alterations in methylation patterns is needed for our hypothesis to be plausible in the classic view of the meaning of evolutionary change.

SPECULATIONS ON THE EVOLUTIONARY IMPLICATIONS OF EARLY EXPOSURE TO ENVIRONMENTAL ESTROGENS

Holliday (1998) proposed that teratogens could target mechanisms that control patterns of DNA methylation in particular regions of the genome of developing embryos, modifying methylation patterns of the same DNA sequence in somatic cells, leading to a developmental alteration, and subsequently producing changes in germ line cells. Moreover, if such altered methylation patterns are eventually transmitted to a subsequent generation, the same type of defect might be seen (Holliday 1998). Phytoestrogens could act in the same manner, but with the peculiarity that they are naturally available for consumption by many organisms. Phytoestrogens are present in high quantities in food items commonly included in the natural dietary composition of rodents, such as fruits, nuts, seeds (Liggins et al. 2000) and especially wheat, oats, and soy (Thigpen et al. 1999).

In this sense, if a natural population of rodents is suddenly subjected to a high intake of phytoestrogens, it is feasible to hypothesize that such a high intake by pregnant rodents could influence the normal reproductive process, altering the mother's hormonal status, the intrauterine signaling, and, consequently, the establishment of DNA methylation patterns in embryos. The resulting phenotypes will be in accordance with the particular pattern of DNA methylation achieved as a consequence of the environmental stimuli, represented in this case by phytoestrogens. As a result, such changes in methylation patterns will persist in the population if the organisms are constantly subjected to this same environmental input and consequently, the achieved phenotypes will also persist throughout generations. Nevertheless, it is important to point out that such a newly formed phenotype must not be considered to be associated with any adaptive goal; on the contrary, the new forms of organisms will fit within the environment they live, resulting from an environmental input that leads to standardized phenotypes in concordance with the environmental stimuli that produced them. In the particular case of imprinted genes, changing methylation patterns on those genes could imply transgenerational persistence of epigenetic changes in the absence of the environmental input that initially produced them.

Because the early stages of ontogeny play key roles in the establishment of phenotypic variation, it is important to determine how environmental signals (particularly CEA) are involved in the developmental process. Nevertheless, a complete understanding of this involvement is difficult at this time. One of the complications is that the mechanisms through which estrogen and CEA bring about physiological actions are not yet clearly understood (Nilsson et al. 2001). Further studies on the effects of CEA on organisms, especially during early stages of ontogeny, are needed to provide new insights, and to help in the understanding of the impact of this class of compounds on ecosystems in general (McLachlan 2001), and, particularly, on the physiologically relevant evolutionary processes that guide the formation of organisms and lineages.

Acknowledgments

The authors wish to thank Roxanna Gomez, Paula Neill, and Renee Hill for their help in the preparation of the manuscript and linguistic revision. We also thank Dr. Gloria Calaf for her critical review of the manuscript and valuable suggestions. Carlos Guerrero-Bosagna gratefully acknowledges CONICYT and also INTA and Nestlé Chile S.A. for the doctoral grant.

REFERENCES

Allen, D., Mitchner, N., Uveges, T., Nephew, K., Khan, S., and Jonathan, N. 1997. Cell-specific induction of *c-fos* expression in the pituitary gland by estrogen. *Endocrinology* 138: 2128–2135.

Amzallag, G. N. 2000. Connectance in sorghum development: beyond the genotype–phenotype duality. *BioSystems* 56: 1–11.

Atchley, W. 1987. Developmental quantitative genetics and the evolution of ontogenies. *Evolution* 41: 316–330.

Bakin, A., and Curran, T. 1999. Role of DNA methylcytosine transferase in cell transformation by *fos*. *Science* 283: 387–390.

Barrett, C., Wong, A., and McLachlan, J. 1981. Diethylstilbestrol induces neoplastic transformation without measurable gene mutation at two loci. *Science* 212: 1402–1404.

Bernardo, J. 1996. Maternal effects in animal ecology. *Am. Zool.* 36: 83–105.

Bestor, T. 2000. The DNA methyltransferases of mammals. *Hum. Mol. Genet.* 9: 2395–2402.

Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation. *Nature* 321: 209–213.

Boettger-Tong, H., et al. 1998. A case of a laboratory animal feed with high estrogenic activity and its impact on in vivo responses to exogenously administered estrogens. *Environ. Health Perspect.* 106: 369–373.

Cairns, J., Overbaugh, J., and Miller, S. 1988. The origin of mutants. *Nature* 335: 142–145.

Caspary, T., Cleary, M. A., Perlman, E. J., Zhang, P., Elledge, S. J., and Tilghman, S. M. 1999. Oppositely imprinted genes p57(Kip2) and igf2 interact in a mouse model for Beckwith–Wiedemann syndrome. *Genes Dev.* 13: 3115–3124.

Clark, M., and Galef, B. 1995. Prenatal influences on reproductive life-history strategies. *Trends Ecol. Evol.* 10: 151–153.

Clark, M., and Galef, B. 1998. Perinatal influences on the reproductive behavior of adult rodents. In T. A. Mousseau and C. W. Fox (eds.). *Maternal Effects as Adaptations*. Oxford University Press, New York, pp. 261–271.

Clark, M., Karpluk, P., and Galef, B. 1993. Hormonally mediated inheritance of acquired characteristics in Mongolian gerbils. *Nature* 364: 712–712.

Constância, M., Pickard, B., Kesley, G., and Reik, W. 1998. Imprinting mechanism. *Genome Res.* 8: 881–900.

Costello, J., and Plass, C. 2001. Methylation matters. *J. Med. Gen.* 38: 285–303.

Coulondre, C., and Miller, J. 1978. Molecular basis of base substitution hotspots in *Escherichia coli*. *Nature* 274: 775–780.

Croteau, R., Kutchan, T., and Lewis, N. 2000. Natural products (secondary metabolites). In B. Buchanan, W. Gruissem, and R. Jones (eds.). *Biochemistry and Molecular Biology of the Plants*. Courier Companies Inc., USA, pp. 1250–1318.

Danzo, B. 1998. The effects of environmental hormones on reproduction. *Cell. Mol. Life Sci.* 54: 1249–1264.

Das, S., Tan, J., Raja, S., Halder, J., Paria, B., and Dey, S. 2000. Estrogen targets genes involved in protein processing, calcium homeostasis, and wnt signaling in the mouse uterus, independent of estrogen receptor-α and -β. *J. Biol. Chem.* 275: 28834–28842.

Day, J., et al. 2002. Genistein alters methylation patterns in mice. *J. Nutr.* 132.(suppl.): 2419S–2423S.

Dean, W., et al. 1998. Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* 125: 2273–2282.

Deng, J., and Szyf, M. 1998. Multiple isoforms of DNA methyltransferase are encoded by the vertebrate cytosine DNA methyltransferase gene. *J. Biol. Chem.* 273: 22869–22872.

Edinger, R., Mambo, E., and Evans, M. 1997. Estrogen-dependent transcriptional activation and vitellogenin gene memory. *Mol. Endocrinol.* 11: 1985–1993.

Endler, J. 1986. *Natural Selection in the Wild*. Princeton University Press, Princeton, NJ.

Ferguson-Smith, A., and Surani, M. A. 2001. Imprinting and the epigenetic asymmetry between paternal genomes. *Science* 293: 1086–1089.

Futuyma, D., and Moreno, G. 1988. The evolution of ecological specialization. *Annu. Rev. Ecol. Systems* 19: 207–233.

Gallo, D., et al. 1999. Reproductive effects of dietary soy in female Wistar rats. *Food. Chem. Toxicol.* 37: 493–502.

Garcia, E., Lacasa, D., and Giudicelli, Y. 2000. Estradiol stimulation of c-*fos* and c-*jun* expressions and activator protein-1 deoxyribonucleic acid binding activity in rat white adipocyte. *Endocrinology* 141: 2837–2846.

Gardiner-Garden, M., and Frommer, M. 1987. CpG islands in vertebrate genomes. *J. Mol. Biol.* 196: 261–282.

Gould, S. J., and Lewontin, R. C. 1979. The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. *Proc. Roy. Soc. Lond.* B205: 581–598.

Hajkova, P., et al. 2002. Epigenetic reprogramming in mouse primordial germ cells. *Mech. Dev.* 117: 15–23.

Hata, K., Okano, M., Lei, H., and Li, E. 2002. Dnmt3L cooperates with the Dnmt3 family of *de novo* DNA methyltransferases to establish maternal imprints in mice. *Development* 129: 1983–1993.

Holliday, R. 1987. The inheritance of epigenetic defects. *Science* 238: 163–170.

Holliday, R. 1998. The possibility of epigenetic transmission of defects induced by teratogens. *Mutat. Res.* 422: 203–205.

Howell, C. Y., et al. 2001. Genomic imprinting disrupted by a maternal effect mutation in the Dnmt1 gene. *Cell* 104: 829–838.

Hyder, S., Stancel, G., Nawaz, Z., McDonnell, D., and Loose-Mitchell, D. 1992. Identification of an estrogen response element in the 3′- flanking region of the murine c-*fos* protooncogene. *J. Biol. Chem.* 267: 18047–18054.

Jablonka, E., Goitein, R., Sperling, K., Cedar, H., and Marcus, M. 1987. 5-aza-C-induced changes in the time of replication of the X chromosomes of *Microtus agrestis* are followed by non-random reversion to a late pattern of replication. *Chromosoma* 95: 81–88.

Jablonka, E., and Lamb, M. 1995. *Epigenetic Inheritance and Evolution, the Lamarkian Dimension*. Oxford University Press Inc., New York.

Jaenisch, R., and Bird, A. 2003. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat. Genet.* 33.(suppl.): 245–254.

Jones, A., and Takai, D. 2001. The role of DNA methylation in mammalian epigenesis. *Science* 293: 1068–1070.

Kamiya, K., Sato, T., Nishimura, N., Goto, Y., Kano, K., and Iguchi, T. 1996. Expression of estrogen receptor and proto-oncogene messenger ribonucleic acids in reproductive tracts of neonatally diethylstilbestrol-exposed female mice with or without post-pubertal estrogen administration. *Exp. Clin. Endocrinol. Diabetes* 104: 111–122.

Khosla, S., Dean, W., Brown, D., Reik, W., and Feil, R. 2001. Culture of preimplantation mouse embryos affects development and the expression of imprinted genes. *Biol. Reprod.* 64: 918–926.

Kierzenbaum, A. 2002. Genomic imprinting and epigenetic reprogramming: unearthing the garden of forking paths. *Mol. Reprod. Dev.* 63: 269–272.

Laird, P., and Jaenisch, R. 1996. The role of DNA methylation in cancer genetics and epigenetics. *Annu. Rev. Genet.* 30: 441–464.

Levy, J., Faber, K., Ayyash, L., and Hughes, C. 1995. The effect of prenatal exposure to the phytoestrogen genistein on sexual differentiation in rats. *Proc. Soc. Exp. Biol. Med.* 208: 60–66.

Li, S., et al. 1997. Developmental exposure to diethylstilbestrol elicits demethylation of estrogen-responsive lactoferrin gene in mouse uterus. *Cancer Res.* 57: 4356–4359.

Liggins, J., Bluck, J., Runswick, S., Atkinson, C., Coward, W., and Bingham, S. 2000. Daidzein and genistein content of fruits and nuts. *J. Nutr. Biochem.* 11: 326–331.

Liu, K., Wang, Y. F., Cantemir, C., and Muller, M. 2003. Endogenous assays of DNA methyltransferases: evidence for differential activities of DNMT1, DNMT2, and DNMT3 in mammalian cells in vivo. *Mol. Cell. Biol.* 23: 2709–2719.

Lyn-Cook, B. D., Blann, E., Payne, P. W., Bo, J., Sheehan, D., and Medlock, K. 1995. Methylation profile and amplification of proto-oncogenes in rat pancreas induced with phytoestrogens. *Proc. Soc. Exp. Biol. Med.* 208: 116–119.

Mac Phee, D. 1998. Epigenetics and epimutagens: some new perspectives on cancer, germ line effects and endocrine disrupters. *Mutat. Res.* 400: 369–379.

Maturana-Romesin, H., and Mpodozis, J. 2000. The origin of species by means of natural drift. *Rev. Chil. Hist. Nat.* 73: 261–300.

McClintock, B. 1984. The significance of responses of the genome to challenge. *Science* 226: 792–801.

McEvoy, T. G., Robinson, J. J., Ashworth, C. J., Rooke, J. A., and Sinclair, K. D. 2001. Feed and forage toxicants affecting embryo survival and fetal development. *Theriogenology* 55: 113–129.

McLachlan, J. 2001. Environmental signalling: what embryos and evolution teach us about endocrine disrupting chemicals. *Endocr. Rev.* 22: 319–341.

Morison, I. M., Croydon, J. P., and Cleverly, S. D. 2001. The imprinted gene and pattern-of-origin effect database. *Nucleic Acids Res.* 29: 275–276.

Milligan, S. R., Balasubramanian, A. V., and Kalita, J. C. 1998. Relative potency of xenobiotic estrogens in an acute in vivo mammalian assay. *Environ. Health Perspect.* 106: 23–26.

Nadal, A., Ropero, A., Laribi, O., Maillet, M., Fuentes, E., and Soria, B. 2000. Nongenomic actions of estrogens and xenostrogens by binding at a plasma membrane receptor unrelated to estrogen receptor α and estrogen receptor β. *Proc. Natl. Acad. Sci. USA* 97: 11603–11608.

Nagao, T., Yoshimura, S., Saito, Y., Nakagomi, M., Usumi, K., and Ono, H. 2001. Reproductive effects in male and female rats of neonatal exposure to genistein. *Reprod. Toxicol.* 15: 399–411.

Nanney, D. L. 1985. Heredity without genes: ciliate explorations of clonal heredity. *Trends Genet.* 1: 295–298.

Newbold, R., Hanson, R. B., Jefferson, W. N., Bullock, B. C., Haseman, J., and McLachlan, J. A. 2000. Proliferative lesions and reproductive tract tumors in male descendants of mice exposed developmentally to diethylstilbestrol. *Carcinogenesis* 21: 1355–1363.

Nijhout, F. H., Wray, G. A., Kremen, C., and Teragawa, K. 1986. Ontogeny, phylogeny and evolution or form: an algorithmic approach. *Systems Zool.* 35: 445–457.

Nilsson, S., et al. 2001. Mechanisms of estrogen action. *Physiol. Rev.* 81: 1535–1565.

Paria, B., Huet-Hudson, Y., and Dey, S. 1993. Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. *Proc. Natl. Acad. Sci. USA* 90: 10159–10162.

Paria, B., Lim, H., Wang, X., Liehr, J., Das, S., and Dey, S. 1998. Coordination of differential effects of primary estrogen and catecholestrogen on two distinct targets mediates embryo implantation in the mouse. *Endocrinology* 139: 5235–5246.

Pino, A., Valladares, L., Palma, M., Mancilla, A., Yañez, M., and Albala, C. 2000. Dietary isoflavones affect sex hormone-binding globulin levels in postmenopausal women. *J. Clin. Endocr. Metab.* 85: 2797–2800.

Razin, A., and Cedar, H. 1994. DNA methylation and genomic imprinting. *Cell* 77: 473 476.

Reik, W., Dean, W., and Walter, J. 2001. Epigenetic reprogramming in mammalian development. *Science* 293: 1089–1092.

Roemer, I., Reik, W., Dean, W., and Klose, J. 1997. Epigenetic inheritance in the mouse. *Curr. Biol.* 7: 277–280.

Rutherford, S. L., and Lindquist, S. 1998. Hsp90 as a capacitor for morphological evolution. *Nature* 396: 336–342.

Santell, R. C., Chang, Y. C., Muralee, G. N., and Helferich, W. G. 1997. Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. *J. Nutr.* 127: 263–269.

Singal, R., and Ginder, G. 1999. DNA methylation. *Blood* 93: 4059–4070.

Steele, F., and Jinks-Robertson, S. 1992. An examination of adaptive reversion in *Saccharomyces cerevisiae*. *Genetics* 132: 9–21.

Surani, M. A. 2001. Reprogramming of genome function through epigenetic inheritance. *Nature* 414: 122–128.

Sved, J., and Bird, A. 1990. The expected equilibrium of the CpG dinucleotide in vertebrate genomes under a mutation model. *Proc. Natl. Acad. Sci. USA* 87: 4692–4696.

Takai, Y., et al. 2000. Preimplantation exposure to bisphenol A advances postnatal development. *Reprod. Toxicol.* 15: 71–74.

Thigpen, J., et al. 1999. Phytoestrogen content of purified, open- and closed-formula laboratory animal diets. *Lab. Anim. Sci.* 49: 530–536.

Vandenbergh, J., and Huggett, C. 1994. Mother's prior intrauterine position affects the sex ratio of her offspring in house mice. *Proc. Natl. Acad. Sci. USA* 91: 11055–11059.

Waddington, C. H. 1952. Genetic assimilation of an acquired character. *Evolution* 7: 118–126.

Waddington, C. H. 1959. Canalization of development and genetic assimilation of acquired characters. *Nature* 183: 1654–1655.

Wake, D., and Larson, A. 1987. Multicellular analysis of evolving lineage. *Science* 238: 42–48.

West-Eberhard, M. J. 1998. Evolution in the light of developmental and cell biology, and *vice versa*. *Proc. Natl. Acad. Sci. USA* 95: 8417–8419.

West-Eberhard, M. J. 2003. *Developmental Plasticity and Evolution*. Oxford University Press, New York.

Weismann, A. 1893. *The Germ-Plasm: A Theory of Heredity*. AMS Press Inc., New York.

Wright, S. 1945. Genes as physiological agents. General considerations. *Am. Nat.* 74: 289–303.

Wu, Q., Ohsako, S., Ishimura, R., Suzuki, J. S., and Tohyama, C. 2004. Exposure of mouse preimplantation embryos to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters the methylation status of imprinted genes *H19* and *Igf2*. *Biol. Reprod.* 70: 1790–1797.

Yoder, J. A., Soman, N. S., Verdine, G. L., and Bestor, T. H. 1997. DNA (cytosine-5)-methyltransferases in mouse cells, and tissues. Studies with a mechanism-based probe. *J. Mol. Biol.* 270: 385–395.

Yokochi, T., and Robertson, K. D. 2002. Preferential methylation of unmethylated DNA by mammalian *de novo* DNA methyltransferase Dnmt3a. *J. Biol. Chem.* 277: 11735–11745.

Yu, O., et al. 2000. Production of the isoflavones genistein and daidzein in non-legume dicot and monocot tissues. *Plant Physiol.* 124: 781–793.

III

Maternal Dietary Consumption of Phytoestrogens and its Effects of on Life-History and Morphometric Characters in Mice Offspring

Running Title: Phytoestrogens and Maternal Effects

Carlos Guerrero-Bosagna,[1,2*] Fernanda Valdovinos,[2] Pablo Sabat[2,3] and Luis Valladares.[1]

[1]Laboratorio de Hormonas y Receptores. Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, Santiago, Chile.

[2]Laboratorio de Ecofisiología Animal. Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago Chile.

[3]Center for Advanced Studies in Ecology & Biodiversity and Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile.

*Author for correspondence: Tel: +56-2-6781435, Fax: +56-2-2214030.

E-mail: catelo@uec.inta.uchile.cl

Words in Manuscript (main text): 2677

ABSTRACT

In mammals, variations in maternal hormonal status are capable of affecting the microenvironment where the fetus develops and its further ontogenetic process. Exogenous extrogens as phytoestrogens could alter the hormonal intrauterine environment, affecting early processes in the embryo responsible for adult life-history and morphologic characters. This paper investigate the effect of feeding adult mice with high amounts of dietary phytoestrogens (soy isoflavones) on morphometric aspects (size, weight and and ano-genital distance) and life-history traits (sex ratio and female sexual maturation) in the offspring. Maternal treatment with dietary isoflavones i) advanced puberty in female offspring, ii) did not produce differences in sex ratio in litters, iii) generate male pups heavier when adults, and iv) did not produce effect on size or ano-genital distance in pups of any gender. The effects of environmental estrogens in mammals open intriguing questions on the role of these compounds when viewed from ecological or evolutionary perspectives.

INTRODUCTION

It has been described in several organisms (fishes: Lombardi, 1996; insects: Carrière, 1994; mammals: Ims, 1987) that the environment experienced by parents can influence the phenotype of the offspring and alter its life history characters. In addition to nutrients, parents can provide their offspring with factors such as pre-regulated genomes, defensive agents, symbionts, pathogens, hormones, enzymes and cultural learning (Rossiter, 1996). The consequences of maternal effects might be seen at many levels of interaction between organisms, among them population dynamics, life history and evolution of characters (Rossiter, 1996; Bernardo, 1996).

In mammals, some maternal effects, such as the consequences of variations in hormonal status, are capable of affecting the microenvironment where the fetus develops (Clark & Galef, 1998) and consequently, its further ontogenetic process (Bernardo, 1996). For example, prenatal exposure to high levels of testosterone induces variation in life history characters such as age at puberty, period of fertility and sex proportion in litters (Clark & Galef, 1995). In parallel, variations in life history characters can have populational consequences, through changes in growth rate or fitness (Futuyma, 1998).

Acting early during development, exogenous estrogenic compounds may have a role producing epigenetic modification during key stages of the ontogeny in mammals (Guerrero-Bosagna et al. 2005) which may alter characters related to life-history traits. Timing of sexual maturation is one of those traits. Howdeshell et al. (1999) showed that prenatal treatment with the synthetic estrogen bisphenol-A significantly reduces the number of days between vaginal opening and first vaginal estrous. Morphological alterations can

also be produced in adults due to exposure to estrogenic compounds early during ontogeny. Takai et al. (2001) have shown that blastocysts exposed to the synthetic estrogen Bisphenol-A produce adult mice that are heavier at weaning than controls, despite having similar weight at birth. Natural estrogenic compounds, such as genistein, have also been studied, but apparently produce the opposite effect on body mass. For example, Nagao et al. (2001) reported that rodents fed with the phytoestrogen genistein between 1 and 5 days post-natal (dpn) give birth to pups with lower post-puberty body weights, as compared with controls.

Sex ratio has been reported to be influenced by environmental cues in both invertebrates and non-mammalian vertebrates (Dyson and Hurst, 2004; Crews, 2003). Exogenous estrogens play an important role in sex determination and together with incubation temperature they may synergetically induce ovarian differentiation in the red-eared slider turtle (Crews, 2003). Nevertheless, also mammals can be subjected to environmental influences in the mechanisms of sex determination. In a recent review, Rosenfeld and Roberts (2004) presented several examples of sex ratio changes in response to nutritional or other environmental factors. They also proposed two main mechanisms implicated in skewing sex ratio: (i) those operating prior to conception and (ii) those favoring one sex over the other after fertilization.

Hormones seem to be very important in biasing sex proportion in mammals. Studies by Clark et al. (1991) reported in Mongolian gerbils (*Meriones unguiculatus*) that female fetuses *in utero* neighbored by two males are exposed to higher levels of testosterone than those *in utero* between two female fetuses. In addition, such differences in the early

exposition to hormones lead to differences in the sex proportion of the offspring. Clark et al. (1993) reported a greater proportion of males in litters born to females surrounded by two males when they were fetuses, than in litters born to females surrounded by two females when fetuses.

The hormonal state of a mammalian female can be strongly influenced by the environment through compounds that are naturally found in her diet (Nagao et al., 2001) and also by xenobiotics (Danzo, 1998). It has been reported that feed toxicants and dietary imbalances of specific nutrients can alter the composition of oviductal and uterine secretions (Mc Evoy et al., 2001). For example, Guay et al (2002) showed in pigs that supplementation of folic acid in maternal diet reduced the homocysteine content in uterine flush by approximately 30% during early pregnancy (preimplantation blastocyst stage). Since maternal mammalian consumption of phytoestrogens could alter the hormonal intrauterine environment where foetus develops (Guerrero-Bosagna et al., 2005), they may also epigenetically alter in the embryo early processes responsible for adult characters, such as life history and morphologic characters.

In the present paper, we investigated the effect of feeding adult mice with high amounts of dietary phytoestrogens on morphometric and life-history characters in the offspring. Morphometric aspects evaluated in the offspring were size, weight and ano-genital distance, while life-history characters studied were sex ratio in litters and female sexual maturation.

MATERIAL AND METHODS

Adult mice (*Mus musculus*) of C3H strain from a lab stock population, reared for several generations, were initially raised in individual plastic cages with a standard laboratory chow diet for rodents (Champion® S.A., Santiago, Chile), the control diet, and water *ad libitum*. Then mice were randomly assigned in male/female couples to one of following experimental treatments: mice were fed with i) control diet or ii) control diet plus a commercial concentrate of soy isoflavones (Soy Life®, Netherlands B. V.) added at 2% (w/w), diet named as ISF. In order to the ensure high levels of plasmatic isoflavones in mice feeding ISF diet, treatment with the ISF diet was initiated two weeks before placing the male and female couples in the same cage. The proportion of the soy isoflavones concentrate in diet was chosen considering a previous study of Gallo et al. (1999), which demonstrated that post weaning long-term consumption of meals with as high as 2.4% soy extract produces significant agonistic effects in a variety of estrogen-dependent tissues and reproductive parameters in female rats and also advances the age of vaginal opening. In both experimental treatments, animals were fed *ad libitum* and maintained at a light cycle of 12:12 at 22 ± 2 °C. In order to avoid maternal cannibalism, pups were not handled until the age of 7 dpn when gender was identified. The time of the onset of vaginal opening, expressed as days after birth, was used to measure sexual maturation in females. All female pups were examined daily after the age of 20 dpn to check the occurrence of vaginal opening. Morphometric parameters such as weight, size and ano-genital distance were measured in all pups at 7, 14, 21 and 42 dpn. Two tailed Student *t* test were used for all the comparisons performed in this study. Differences were considered to be significant at $P<0.05$. All protocols used in the present study were approved by the Institutional Animal

Care and Use Committee at INTA (Instituto de Nutrición y Tecnología de los Alimentos), Universidad de Chile.

RESULTS

No difference was found in sex ratio or litter size between the control and ISF groups. Average sex ratio of males found in litters born to parents treated with the ISF diet was 49, 4 % ± 5.8 (n = 12) and in litters born to parents fed on control diet was 53,2 % ± 4.6 (n = 13) (values expressed as means of % of males per litter ± SE; P=0.63). Litter size values were 8.5 ± 0.58 (n= 13) for control group and 8.1 ± 0.69 (n= 12) for the ISF group (values expressed as means of litter size ± SE; P= 0.68).

Sexual maturation was advanced by approximately 6 days in female pups born to ISF-fed mothers, with vaginal opening occurring at 25.7 dpn ± 0.48 (n= 32) as compared to 31.6 dpn ± 0.75 (n= 39) in the control group (values expressed as means of day of vaginal opening ± SE; Fig. 1a and 1b; P<0.001). Not surprisingly, weight, size and ano-genital distance in females at the day of vaginal opening were also reduced in the ISF feeding group. Weight changed from 17.9 g ± 0.11 (n= 39) to 12.6 g ± 0.09 (n= 32), size from 8.1 cm ± 0.08 (n= 39) to 7.2 cm ± 0.14 (n= 32), and ano-genital distance from 6.6 mm ± 0.45 (n= 39) to 5.8 mm ± 0.54 (n= 27) (all values are expressed as means ± SE; P<0.001 for all the comparisons). Such decreases are certainly associated with the reduced age at which females mature.

Figure 1a

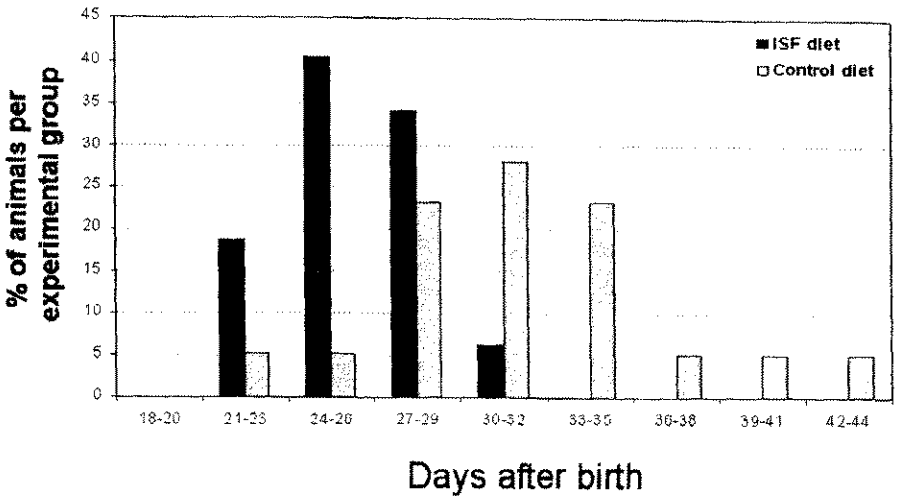


Figure 1b

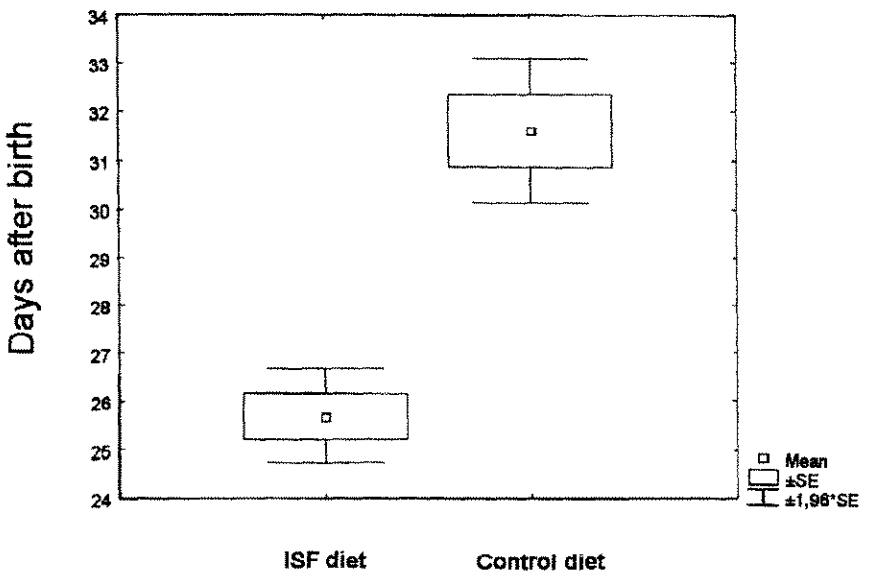


Fig 1 – Timing of female mice sexual maturation expressed as (**a**) distribution of occurrence of vaginal opening along days after birth and as (**b**) means of days of occurrence of vaginal opening after birth ± SE ± 1,96*SE. Differences were tested with Student *t* test and considered to be significant at $p<0.05$.

Male pups born to mothers in the control group were heavier than those born to mothers in the ISF group at the age of 42 dpn (Table 1); nevertheless, no difference in weight for females was observed between the two groups (Table 2). Furthermore, there were no differences in weight among male and female pups within the ISF group at the age of 42 dpn (P=0.30), but within the control group, males were heavier than females (Fig. 2; P<0.001). No differences were detected among the experimental groups in size or ano-genital distance for either males (Table 1) or females (Table 2).

Table 1: Morphometric parameters in male pups measured at days 7, 14, 21 and 42 after birth. Values are expressed as means of the average male values per litter ± SE. Differences among groups (*) were tested with Student t test and considered to be significant at $P<0.05$.

		day 7 dpn			day 14 dpn			day 21 dpn			day 42 dpn		
		Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Average Pup Weight per Litter (g)	ISF	4.22	0.25	9	6.34	0.44	9	9.29	0.67	9	23.85	0.96	9
	Control	4.45	0.28	12	6.72	0.26	12	9.55	0.57	12	26.18	0.56	12
	P values	0.5094			0.5122			0.7691			0,039*		
		Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Average Pup Size per Litter (cm)	ISF	4.61	0.1	9	5.69	0.15	9	6.41	0.18	9	8.96	0.19	9
	Control	4.74	0.07	12	5.84	0.11	12	6.74	0.15	12	9.34	0.12	12
	P values	0.3036			0.4217			0.1727			0.0885		
		Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Average Pup Ano-Genital Distance per Litter (mm)	ISF	4.05	0.15	9	6.09	0.16	9	8.91	0.4	9	16.7	0.43	9
	Control	4.31	0.14	12	6.35	0.24	12	9.41	0.44	12	16.94	0.38	12
	P values	0.2276			0.1997			0.4281			0.7018		

Table 2: Morphometric parameters in female pups measured at days 7, 14, 21 and 42 after birth. Values are expressed as means of the average male values per litter ± SE. Differences among groups (*) were tested with Student t test and considered to be significant at $P<0.05$.

		day 7 dpn			day 14 dpn			day 21 dpn			day 42 dpn		
		Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Average Pup Weight per Litter (g)	ISF	4.17	0.25	10	6.16	0.36	10	9.11	0.62	10	22.52	0.81	10
	Control	4.46	0.2	12	6.83	0.25	12	9.2	0.47	12	22.72	0.32	12
	P values	0.3931			0.1148			0.907			0.8087		
		Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Average Pup Size per Litter (cm)	ISF	4.61	0.11	10	5.59	0.14	10	6.44	0.2	10	8.83	0.14	10
	Control	4.74	0.08	12	5.87	0.09	12	6.72	0.15	12	9.07	0.11	12
	P values	0.3306			0.0864			0.2637			0.1874		
		Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Average Pup Ano-Genital Distance per Litter (mm)	ISF	2.41	0.14	10	3.94	0.15	10	4.9	0.12	10	7.06	0.09	10
	Control	2.71	0.17	12	3.92	0.14	12	5.13	0.18	12	7.12	0.14	12
	P values	0.2029			0.9241			0.331			0.7342		

Figure 2

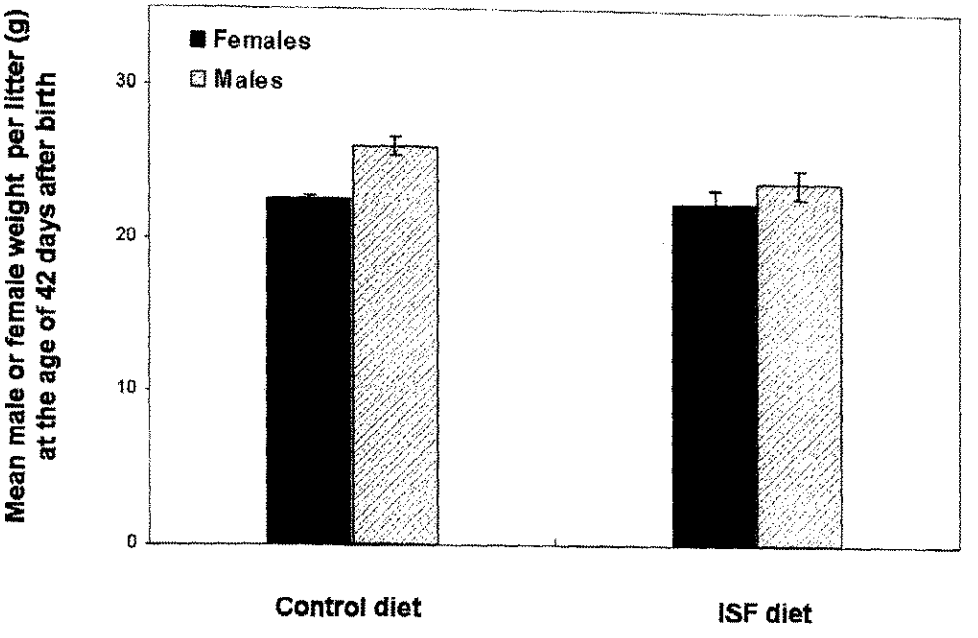


Fig 2 –Comparison of weight at day 42 after birth between female and male pups belonging to ISF or Control group. Values are expressed as means of the average male or female weights per litter ± SE. Differences (*) were tested with Student *t* test and considered to be significant at $p<0.05$.

DISCUSSION

The present study evaluated aspects related to morphology and life-history traits in the offspring of an experimental population of mice subjected to a high dietary consumption of isoflavones. Regarding morphometric aspects, we found no effect of the parental treatment with dietary isoflavones on size or ano-genital distance in pups. Nevertheless, we found that male pups born within the control group were heavier at the age of 42 dpn than those born within the ISF group. This is concordant with experiments performed by Nagao et al. (2001) showing lower body weight in mice pups born to mothers injected with the phytoestrogen genistein. Nevertheless, our results show the opposed effects of those reported by Takai et al. (2000), in which culturing blastocytes in presence of the synthetic estrogen Bisphenol-A resulted in heavier pups at weaning when compared with controls. This could be due to the source of the estrogen (natural or synthetic) employed in these studies. It is likely that environmental estrogens, such as genistein, would produce effect others than those produced with synthetic estrogens, such as Bisphenol-A.

The mechanism behind these weight differences could have either an epigenetic or a physiological base, given that after birth, pups were fed on the same diet as parents. Interestingly, in the present study the same effect was not observed in females. Therefore, if the mechanism behind such weight difference is epigenetic, imprinted genes could be involved, given their particularity of being differentially methylated in both alleles (Constância et al., 1998).

Nevertheless, other evidence suggests that the mechanism behind this sex specific effect in weight could be merely physiological and related with testosterone levels, as it is well

known that body mass is strongly influenced by testosterone in males. While some studies in human granulose-luteal cell cultures have shown that phytoestrogens inhibit aromatase activity, leading to a reduced conversion from androgens to estrogens (Whitehead and Lacey, 2003; Lacey et al., 2005), other authors show that dietary phytoestrogens could partially prevent the disruption in the spermatogenesis seen in aromatase knocked out (ArKO) male mice (Robertson et al., 2002).

Although both findings may seem contradictory, phytoestrogens could be acting at both levels, in one hand inhibiting aromatase activity and, in parallel, mimicking the estrogen in some tissues. The fact that the effects of dietary phytoestrogens are independent of changes to the pituitary-gonadal axis (Robertson et al., 2002) supports this idea. In addition, the above mentioned incongruence could be related with the type of phytoestrogen employed in each study. Recently, Edmunds et al. (2005) reported that genistein but not other phytoestrogens stimulated aromatase activity in endometrial stromal cells. Further investigation should be done in order to elucidate if these male related response of weight to phytoestrogens are dependent on testosterone production.

It has been hypothesizes that phytoestrogenic influences could take place either directly, through the presence of isoflavones in uterine secretions, or indirectly, mediated by other compounds secreted in the uterine epithelia such as 4-OH-17β-estradiol, but that would be responding to circulating levels of isoflavones (Guerrero-Bosagna et al., 2005). Clark & Galef (1995) proposed that early exposure to differential levels of testosterone induces sex ratio alterations in litters. Based on that tendency, we suspected that feeding female mice

with high amounts of phytoestrogens could alter the uterine microenvironment where the embryo develops and, in consequence, this could have effects on the sex ratio of the offspring. Nevertheless, we did not find significant differences in the percent of males in litters among controls and ISF treated, showing that eventual effects of phytoestrogens do not interfere with the mechanisms of sex determination.

Regarding life-history traits, we found that the maternal treatment with dietary isoflavones advanced puberty in female offspring. This could also be the product of either an epigenetic or a purely physiological phenomenon. It is interesting to note that, in the experiments of Gallo et al (1999), age at vaginal opening was significantly reduced when animals were feeding a 2.4% isoflavone meal after weaning, but not when feeding reduced amounts of isoflavones. This suggests that the effect of advancement in sexual maturation is physiological and strongly dependent on the amount of isoflavones in the diet. Nevertheless, we can not discard that an epigenetic effect triggered during prenatal stages of ontogeny is also playing a role in producing an earlier sexual maturation. Further studies should be performed in order to clarify whether these advancements in sexual maturation are only physiological, perhaps triggered when the stimulus is produced after birth and before weaning, or whether they are in fact epigenetically based.

In spite of the mechanism involved in producing advancement of female sexual maturation, important consequences on population structure and/or evolutionary process can emerge from that shift. Compared to other life histories traits, changes in the timing of sexual maturation impact strongly on fitness, across a variety of types of life histories (Stearns, 1992). For example, organisms that mature earlier have a high probability of surviving to

maturity (Bell, 1980). Advanced age of maturity will produce shorter generations, and higher survivor to maturity due to a shorter juvenile period (Stearns, 1992).

The populational effects of earlier sexual maturation have been extensively treated in fishes but not in mammals. Recent evidence in fish shows that evolutionary changes in the maturation reaction norm have strong repercussions for the mean size and the density of harvested individuals, which results, in most of cases, in reduction of biomass (Ernande et al., 2004). Body size is another feature that is influenced by the timing of maturation. Aday et al. (2006) showed in the bluegill (*Lepomis macrochirus*) that body size is influenced by both resource availability and the processes controlling timing of maturation, acting in sex-specific ways. Our results in mice, however, show that adult body weight and size in females were not affected by maternal consumption of phytoestrogens, in spite of the marked variations in the age of vaginal opening. Timing of maturation is usually though to evolve as a consequence of selective pressures, which implies restrictive environmental forces acting on organisms. Nevertheless, this paper emphasizes how environment, rather than restricting the ontogeny of organisms, can act inducing changes in life history traits, which may also lead to ecological and evolutionary changes.

Steroid-mediated maternal effects in viviparous organisms could have long-lasting consequences on those life history aspects related to fitness of the offspring in vertebrates in general and be an important evolutionary factor (Uller et al., 2004). Environmental estrogens should not be placed apart when speaking of steroid mediated maternal effects. The increasing knowledge on the mechanisms of actions of environmental estrogens and their physiological repercussions in mammals open intriguing questions on the role of these

compounds when viewed from ecological or evolutionary perspectives, something that very few studies have explored to date.

REFERENCES

Aday, D. D., Phillip, D. P. & Wahl, D. H. (2006) *Oecologia* **147**, 31-38.

Bell, G. (1980) *Am. Nat.* **116**, 45-76.

Bernardo, J. (1996) *Am. Zool.* **36**: 83-105.

Broer, K. H., Winkhaus, I., Sombroek, H. & Kaiser, R. (1976) *Int. J. Fertil.* **21**, 181–185.

Carrière, Y. (1994) *Heredity* **72**, 420-430.

Clark, M., Crews, D. & Galef, B. G. (1991) *J. Physiol. Behav.* **49**, 239-243.

Clark, M., & Galef, B. (1995) *Trends Ecol. Evol.* **10**, 151–153.

Clark, M. & Galef, B. (1998) in *Maternal effects as adaptations* (Mousseau and Fox eds, Oxford University Press, NY), pp. 261-271.

Clark, M., Karpluk, P. & Galef Jr, G. (1993) *Nature* **364**, 712.

Constância, M., Pickard, B., Kesley, G., & Reik, W. (1998) *Genome Res.* **8**, 881–900.

Crews, D. (1996) *Zool. Sci.* **13**, 1-13

Crews, D. (2003) *Evol. Dev.* **5**, 50-55.

Crews, D., Fleming, A, Willingham, E., Baldwin, R. & Skipper, J. K. (2001) *J. Exp. Zool.* **290**, 597-606

Cui, K. H. (1997) *Mol. Hum. Reprod.* **3**, 61–67.

Dyson, E. A. & Hurst, G. D. (2004) *Proc. Natl. Acad. Sci. USA* **27**, 6520-6523.

Edmunds, K., Holloway, A. C., Crankshaw, D. J., Agarwal, S. K. & Foster, W. G. (2005) *Reprod. Nutr. Dev.* **45**, 709–720.

Ernande, B., Dieckmann and Heino, M. (2004) *Proc. R. Soc. Lond. B* **271**, 415-23.

Futuyma, D. 1998. *Evolutionary Biology* (3rd Edition, Sinauer Associates, Inc. Publishers. Printed in USA).

Gallo, D., Cantelmo, F., Distefano, M., Ferlini, C., Zannoni, G. F., Riva, A., Morazzoni, P., Bombardelli, E., Mancuso, S., Scambia, G. (1999) *Food Chem. Toxicol.* **37**, 493-502.

Guay, F., Matte, J. J., Girard, C. L., Palin, M. F., Giguere, A. and Laforest J. P. (2002) *Br. J. Nutr.* **88**, 253–263.

Guerrero-Bosagna, C. Sabat, P. & Valladares, L. (2005) *Evol. Dev.* **7**, 341-350.

Haqq, C & Donahoe, P. (1998) *Phys. Rev.* **78**, 1-33.

Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G. & vom Saal, F. S. (1999) *Nature* **401**, 763-764.

Ims, R. A. (1987) *Ecology* **68**, 1812-1818.

Lacey, M., Bohday, J., Fonseka, S. M., Ullah, A. I.. & Whitehead, S. A. (2005) *J. Steroid. Biochem. Mol. Biol.* **96,** 279-86.

Lombardi, J. (1996) *Am. Zool.* **36**, 106-115.

Nagao, T., Yoshimura, S., Saito, Y., Nakagomi, M., Usumi, K., & Ono, H. (2001) *Reprod. Toxicol.* **15**, 399–411.

Nishino, F., Hattori, N., Tanaka, S. & Shiota, K. (2004) *J. Biol. Chem.* **21**, 22306-22313.

Robertson, K. M., O'Donnell, L., Simpson, E. R. & Jones M. E. (2002) *Endocrinology* **143**, 2913-2921.

Rosenfeld, C & Roberts, M. (2004) *Biol. Reprod.* **71**, 1063-1070.

Rossiter, M. C. (1996) *Annu. Rev. Ecol. Syst.* **27**, 451-476.

Stearns, S. C. (1992) *The evolution of life histories* (Oxford University Press, NY, Printed in USA).

Takai, Y., Tsutsumi, O., Ikezuki, Y., Kamei, Y., Oruga, Y., Yano, T., Taketan, Y. (2001) *Reprod. Toxicol.* **15**, 71–74.

Uller, T., Massot, M., Richard, M., Lecomte, J. & Clobert, J. (2004) *Evolution* **58**, 2511-2516.

Whitehead, S. A. & Lacey, M. (2003) *Hum. Reprod.* **18**, 487-94.

IV

Sex-specific methylation patterns change in offspring born to mothers consuming a diet rich in phytoestrogens

Running Title: Phytoestrogens and Methylation

Carlos Guerrero-Bosagna,[1,2,3*] Pablo Sabat,[3,4] Luis Valladares,[1] and Susan J Clark[2]

[1]Laboratorio de Hormonas y Receptores. Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, Santiago, Chile.

[2]Epigenetics Laboratory. Garvan Institute of Medical Research, Sydney, Australia.

[3]Laboratorio de Ecofisiología Animal. Departamento de Ciencias Ecológicas, Facultad de Ciencias, Universidad de Chile, Santiago, Chile.

[4]Center for Advanced Studies in Ecology & Biodiversity and Departamento de Ecología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile.

*Author for correspondence: Tel: +56-2-6781435, Fax: +56-2-2214030.

E-mail: catelo@uec.inta.uchile.cl

Words in Manuscript (main text): 4316

ABSTRACT

Many factors and enzymes appear to act together with DNA methyltransferases in the process of DNA methylation. Prenatal period is a crucial stage for the establishment of methylation patterns. Exposure to some chemicals through maternal dietary consumption, such as phytoetrogens, could affect the process of DNA methylation resetting in mammalian embryos. In this paper we analysed in mice the consequences of a maternal consumption a diet (ISF diet) with high doses of phytoestrogens (2% of a soy-isoflavones extract) on methylation profiles in the offspring. We analysed the promoter regions of 3 genes to check for effects on tissue specific gene methylation (skeletal α-Actin and ERα in liver and pancreas) and on global methylation changes (LINE elements). Maternal ISF diet consumption increases offspring levels of methylation in two CpG sites in females and one CpG site in males for α-Actin in liver, and in one CpG sites for Erα in liver. No change was detected for LINE elements in blastocysts. Tissue specific differences in methylation profiles were also detected in the present study. For α-Actin, pancreas shows a different methylation profile as liver, heart, skeletal muscle and kidney. For ERα, we found absence of methylation in pancreas, contrasting with the methylation profile seen in liver. Also interesting is the finding of reduced base change in two CpG sites in LINE elements in blastocysts in response to the maternal treatment with ISF diet. Maternal consumption of compounds as phytoestrogens may influence and bias the process of establishment of methylation patterns in mammalian embryos.

INTRODUCTION

DNA methylation is one of the epigenetic mechanisms regulating gene expression in mammals. Recent evidence, however, shows that DNA methylation is not acting alone in the process of epigenetic regulation and it is now clear that the mechanism behind establishment and maintenance of DNA methylation is complex. RNA factors, histone methylation and chromatin remodelling enzymes appear to act together with DNA methyltransferases for the establishment and maintenance of methylation patterns and for generating site specific methylation and tissue specific differences (Chen & Riggs, 2005).

It is widely known that reprogramming of DNA methylation patterns takes place at least three times during development (reviewed in Chen & Riggs 2005, Guerrero-Bosagna et al. 2005; Li 2002; Reik et al. 2001). Pre-implantation period is one of the key stages in the mammalian development. A general genome-wide demethylation process occurs in that period, beginning after the fecundation, and a further re-establishment of methylation takes place before implantation (Reik et al., 2001). Imprinting genes may be particularly susceptible to methylation changes that occur during pre-implantation development (Khosla et al., 2001), contrasting with non-imprinted genes, that acquire their methylation patterns after implantation (Kafri et al., 1992; Monk et al., 1987). Regardless of the specific time during early development, before or after implantation, the prenatal period is a crucial stage for the establishment of methylation patterns and exposure to some chemical compounds through maternal dietary consumption could have consequences on the process of DNA methylation resetting in mammalian embryos (Guerrero-Bosagna et al., 2005).

Recent papers have shown that early exposure to some chemical compounds can alter methylation in specific genes. Anway at al. (2005) showed that exposing a gestating rat

mother to the endocrine disruptor vinclozine produced altered methylation patterns in 25 different PCR products after a methylation-sensitive restriction enzyme digestion analysis. Among those PCR products, two fragments were identified in the study: one corresponding to a region of the lysophospholipase gene and other corresponding to a region codifying for the cytokine-inducible SH2 protein. Waalkes *et al.* (2004) showed that maternal exposure to arsenic alters methylation in the promoter region of the Estrogen Receptor alpha gene (ERα) in the offspring. Wu *et al.* (2004) demonstrated that pre-implantation embryos exposed to the environmental contaminant 2,3,7,8-Tetrachlorodibenzo-p-dioxin tended to have decreased expression levels of the imprinted genes *H19* and *Igf2*, which correlated with increased methylation in the 430-base pair *H19/Igf2* imprint control region. It has also been shown that a normally methylated CpG site in the promoter of the lactoferrine gene is unmethylated in developmentally estrogenised mice (Li *et al.* 1997).

Phytoestrogens could also contribute to changes in the DNA methylation patterns triggered during early stages of development. Two studies have shown that treating mice with phytoestrogens can alter methylation patterns, however in both cases treatment with phytoestrogens is started after birth. Lyn -Cook *et al.* (1995) showed that treating neo-natal mice with injections of the phytoestrogens Cumestrol and Equol, between days 1-10 post-natal, triggers a hypermethylation of *H-Ras* in the pancreas. Day *et al.* (2002) fed mice with Genistein after 8 wk of age, and also reported changes in methylation patters in several CpG islands of specific genes. To the best of our knowledge, no work has shown that maternal treatment with dietary phytoestrogens could lead to changes in methylation patterns in the offspring.

In the present paper, we studied if treating mice mothers with a diet rich in isoflavones, the methylation patterns observed in two tissue specific genes, the skeletal α-Actin gene and in ERα gene, were altered in the liver or pancreas from the adult offspring. In addition, we evaluated if the maternal consumption of isoflavones also affected methylation patterns in the repeated LINE elements in blastocysts, to determine if global methylation may also be affected by the diet rich in phytoestrogens. We used bisulphite genomic sequencing to detect changes in methylation of individual CpG sites across the promoter regions of the genes (Clark et al., 1994).

MATERIAL AND METHODS

Animals and Experimental Treatments

Adult mice (*Mus musculus*) of a *Balb/c* strain from a lab stock population, reared for several generations, were initially maintained in individual plastic cages with standard laboratory chow diet for rodents (Champion® S.A., Santiago, Chile) and water *ad libitum*. Then, females were randomly assigned to one of the experimental treatments. One treatment consisted of keeping these females mice feeding on the same diet provided previously. In the other treatment, females were subjected to a diet elaborated with the control diet plus a commercial concentrate of isoflavones (Soy Life®, Netherlands B. V.) added at 2% (w/w), which was named ISF diet. One male were assigned to each female and pregnancy was detected by observation of the vaginal plug in the females. In order to ensure high levels of plasmatic isoflavones in the females belonging to the treatment with ISF diet, feeding was initiated two weeks before placing male and female in the same cage.

In both experimental treatments, animals were fed *ad libitum* and maintained at a light cycle of 12:12 at 22 ± 2 °C. Pregnant female mice were used either to obtain blastocysts and also adult pups, from where DNA samples were isolated from liver and pancreas.

Animal Sacrifices

Females used to obtain blastocysts were sacrificed at the fourth day after detection of the vaginal plug. The offspring used to obtain DNA from pancreas and liver were sacrificed after the age of 42 days. All sacrifices were performed according procedures recommended by the 2000 Report of the American Veterinary Medicine Association (AVMA) Panel on Euthanasia (Beaver et al., 2001). Animals were introduced in a glass chamber with CO_2 and maintained until 1 minute after no vital signs were observed. Then, cervical dislocation was practiced to ensure the animal was killed.

Collection of Blastocysts

After sacrifice, pregnant females were dissected, uteri were extracted and both uterine horns were separated. Each one was flushed with M16 medium (Sigma®) and the elutant collected in small glass capsules. Identification of blastocytes was performed under 40X magnification. Using a glass transfer micro-pipette, embryos were placed in micro-drops of M16 medium under a layer of sterile mineral oil (Sigma®) inside polystyrene culture dishes (Nunclon™, Denmark). All blastocysts obtained from each female were pooled in a single micro-drop of medium. Capsules with blastocysts were maintained at -20° C until DNA isolation was performed.

Isolation of Nucleic Acids from Blastocysts and Adult Tissue

DNA from embryos was isolated by the proteinase K method, described by Zuccotti and Monk (1997); 72 µl of a solution containing 280 µg/ml proteinase K, 1mM SDS and 111pg/ml tRNA from E. coli (Sigma®) were added to the micro-drops containing the pooled blastocysts, under the mineral oil. Then, capsules were placed over a thermal plate at 37° C for 90 minutes. Micro-drops containing DNA were transferred to 600 µl tubes and covered with mineral oil. Tubes were incubated at 98° C for 15 minutes. For liver and pancreas from adult mice, DNA was extracted with Wizard® DNA Extraction Kit (Promega), following product directions. All DNA samples were stored at -20° C until analysis of DNA methylation through *Bisulphite Sequencing* was performed.

Bisulphite Treatment of DNA

DNA bisuphite treatment was essentially carried out as previously described by Clark *et al.* (1994) and Clark and Frommer (1997), with modifications described in Warnecke *et al.* (2002). For blastocysts DNA, bisulphite incubation was reduced to 4 h. For adult liver and pancreas, incubation times with bisulphite varied depending on the quality of the DNA samples, previously checked in 0.8% agarose gels. More degraded samples were treated with bisulphite for 4 hrs, partially degraded samples were treated for 6 hrs and intact high molecular weight DNA samples were treated with bisulphite for 16hrs. The bisulphite reaction was desalted using a DNA clean-up column (Promega), as instructed by manufacturer. Bisulphite treated DNA from liver and pancreas was eluted in 50 µl H_2O and DNA samples from blastocysts were eluted in 20 µl H_2O.

PCR conditions

For the skeletal α-Actin promoter, nested primers and PCR conditions used were the same as previously described (Warnecke & Clark, 1999). For the ER-α gene, a fragment in the promoter region of the exon C (Kos *et al.*, 2000) was amplified using the following semi-nested PCR primers: forward external, 5'-GAGTTTTTTTTAGGAATGTTGATTTT-3'; forward internal, 5'-GGAGGGGTTGTTAAGTGTTTT -3'; reverse, 5'-ACACAACTTCCTTCTCCAACTAAAAA-3'. PCR conditions were the same as for α-Actin, except that annealing temperatures used for the first round primers were 63° C and for the second round primers were 59° C. For the LINE elements , semi-nested PCR primers used were: forward, 5'-GGTYGTTATTTTGGTTTTGGGATTT-3'; reverse external 5'-TCAACTTCTATAATACAAACTCTCACCTATACAA-3'; reverse internal, 5'-ACCAAATAACACCTACTACTCCTAAAACTAAAA-3'. PCR conditions for LINE elements were also the same as for α-Actin, except that annealing temperatures used for the first round primers were 61.2° C and for the second round primers were 59.5° C. All PCR reactions for bisulphite treated DNA were set up in a volume of 20 µl containing 1X Promega master mix and forward and reverse primers. All PCRs were performed in triplicate for each tissue sample.

Cloning and Sequencing

For cloning, PCR products in triplicate were pooled for each sample and then purified using Wizard® PCR clean-up columns (Promega). Purified DNA fragments were cloned into the pGEM-T-Easy Vector (Promega) using the Rapid Ligation Buffer System (Promega). For direct sequencing, all triplicate PCR products were purified by the Exo-Sap method

(Exonuclease I and Shrimp Alkaline Phosphatase from Amersham Biosciences). The sequencing reactions for both direct PCR products and clones were carried out by using the Dye Terminator cycle sequencing kit with AmpliTaq DNA polymerase FS (Applied Biosystems) and the automated 373A NA Sequencer (Applied Biosystems).

The methylation profiles were analysed by either direct quantitative bisulphite PCR sequencing or PCR clonal analysis. For α-Actin and ERα, direct PCR bisulphite sequencing analysis was used. All the direct PCR sequencing analyses were performed on raw data electrophenograms, that permits quantification of the differences among methylated and unmethylated peaks at each CpG site. Examples of methylated and unmethylated sequences are shown in the figures (Fig. 1a and 1b). The LINE elements were analysed by cloning and sequencing analysis and quantification of methylation was expressed as percent of methylated sites in clones for each CpG site.

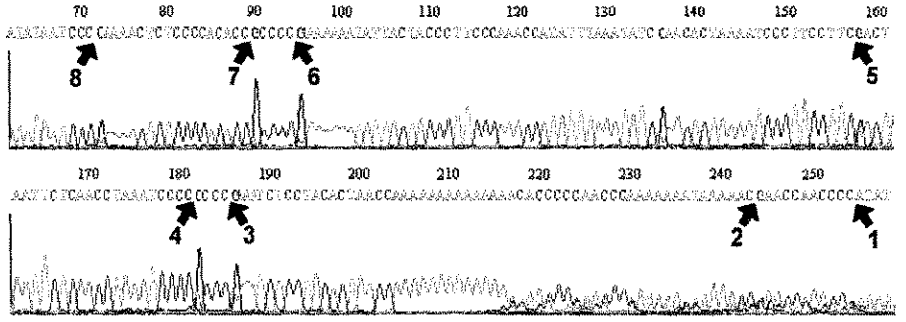
Statistical Analyses

All statistical analyses were performed with Statistica 6.0 (Statsoft®). We used multivariate ANOVA to test for changes in methylation, and either broad or per CpG site effects were assessed using the multivariate or univariate approaches. All differences were considered as significant at $P<0.05$, except when other P is shown.

FIGURES

Figure 1) Sequence of α-Actin

a) Sample of a methylated



b) Sample of an unmethylated

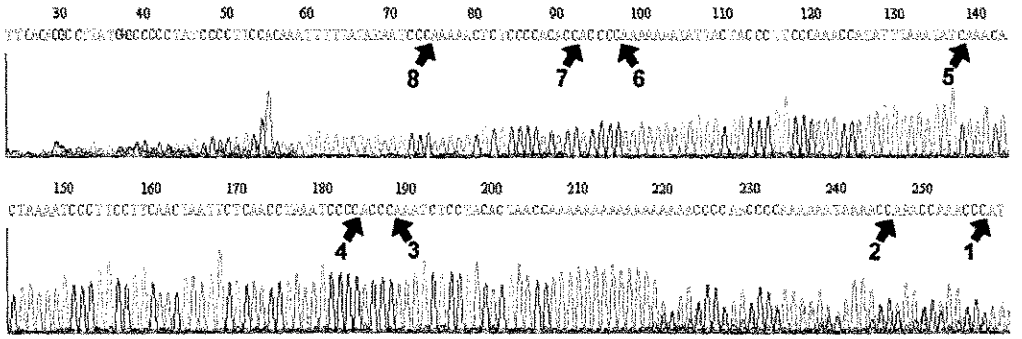


Fig. 1 – Direct sequencing profile of α-Actin PCR products amplified from bisulphite treated DNA. Sequence includes the 8 CpG sites analysed which are shown with arrows in the figure. The shown sequences were obtained with the reverse primer. CpG sites in the forward strand appear in the reverse strand as CG when methylated and as CA when unmethylated. Two samples are shown, one that is majoritary methylated (**a**), one that is completely unmethylated (**b**).

RESULTS

The methylation profile in the offspring born to mice fed on high doses of phytoestrogens was analysed in the promoter regions of 3 genes (skeletal α-Actin, ERα and LINE elements) in order to determine whether there was any effect on tissue specific gene methylation or global methylation changes. Specific gene methylation were analysed in skeletal α-Actin and ERα, and global methylation was studied by changes in methylation of the LINE repeated element.

For the skeletal α-Actin promoter, we determined the methylation profile of 8 CpG sites, using direct bisulphite DNA sequencing across the promoter region, from 529-785 (Fig. 2), in male and female offspring from liver and pancreas. The analysis with multivariate ANOVA showed that no global differences in methylation in liver between DNA from pups born to mothers fed with the control diet plus 2% isoflavones extract (ISF diet) or the control diet, performing comparisons within males, within females or pooling male and female data. Nevertheless, when evaluating the individual CpG site effects, we found that CpG sites # 4 and # 7 showed increased levels of methylation in ISF group versus the control group in liver from females (Fig. 3a). CpG Site # 7 had decreased level of methylation in ISF group versus controls in liver from males (Fig. 3b). No CpG site specific effect was detected pooling male and female data (Fig. 3c).

Figure 2) α–Actin CpG sites analysed

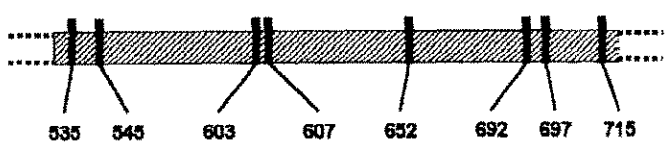


Fig. 2 – Map of the analysed region for α-Actin, showing the locations of CpG sites.

Figure 3

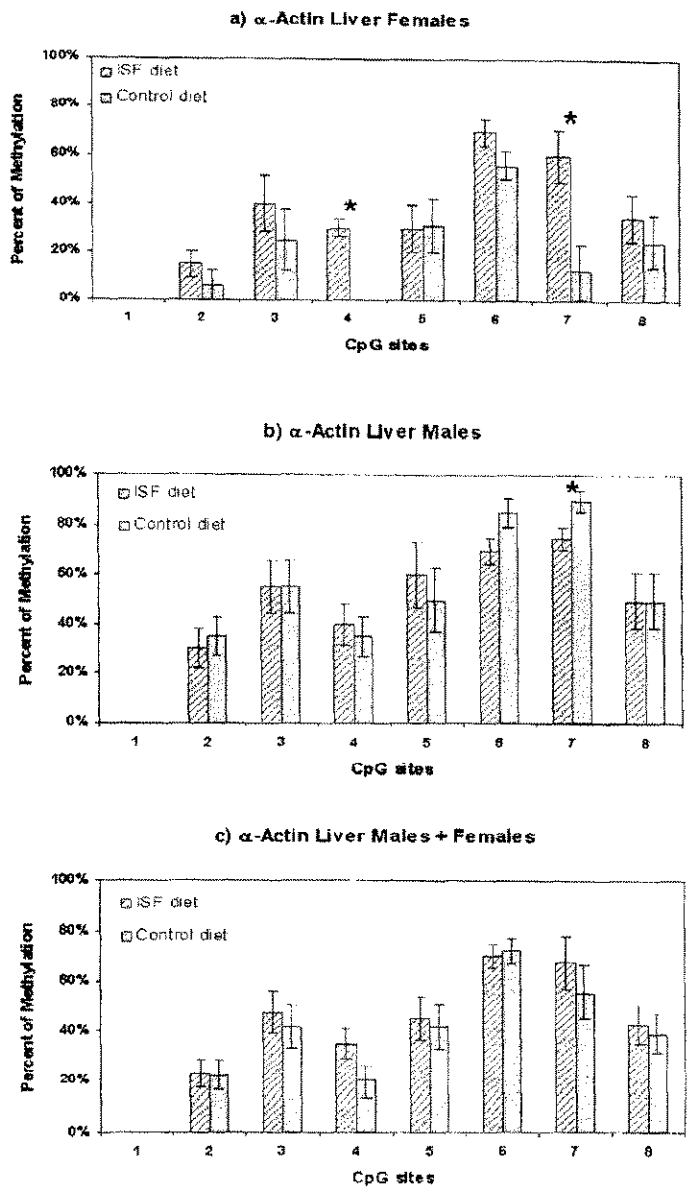


Fig. 3 – Methylation profile of the 8 CpG sites analysed for α-Actin in adult liver from mice born to mothers consuming control or ISF diet. Methylation values per CpG site were calculated measuring the high of methylated or unmethylated peaks in the raw data electrophenograms and then the median of the methylation values in triplicate was estimated for each animal. (**a**) represents comparison between females (n = 4 for control and n = 5 for ISF); (**b**) represents comparison between males (n = 5 for control and n = 5 for ISF); (**c**) represents comparison between control and ISF groups, pooling male and female data (n = 9 for control and n = 10 for ISF). Values are expressed as the means of the methylation values ± SE. * indicates univariate differences detected with with MANOVA and considered to be significant at $P<0.05$.

For pancreas, multivariate analysis revealed no differences between experimental groups, comparing within males or within females. Nevertheless, when performing the univariate analysis, site # 5 showed increased level of methylation in ISF group regarding to controls in males (Fig 4a), although no CpG site specific change were detected for females (Fig. 4b). Interestingly, pooling male and female data together revealed a nearly significant difference with the multivariate approach (P=0.08), suggesting that overall changes in methylation are taking place in α-Actin for pancreas. Moreover, two CpG site specific changes in methylation were detected, in sites # 5 and 8, showing increased levels of methylation in the ISF group with respect to control group (Fig. 4c). The other analysis performed was a comparison in methylation status between pancreas and liver, using only control animals (both males and females), in order to address the occurrence of tissue specific methylation for α–Actin. Both multivariate and univariate differences (sites # 2, 5 and 8) were found in methylation among liver and pancreas, showing a natural occurrence of tissue specific methylation among these tissues for α–Actin (Fig 5).

For ERα alpha, the methylation profile of 6 CpG sites located from -2285 to -1979 were analysed (Fig. 6). In liver, the analysis with multivariate ANOVA showed no broad effects among males or females. Nevertheless, with the univariate analysis a difference was detected between females in CpG site #1, showing an increased level of methylation in mice from ISF group (Fig 7a). No differences were detected with the univariate analysis in males (Fig. 7b) or pooling together male and female data (Fig. 7c). In addition, we also found tissue specific methylation for ERα, where CpG sites were differentially methylated in liver, as shown in fig. 6c, but totally unmethylated in pancreas.

Figure 4

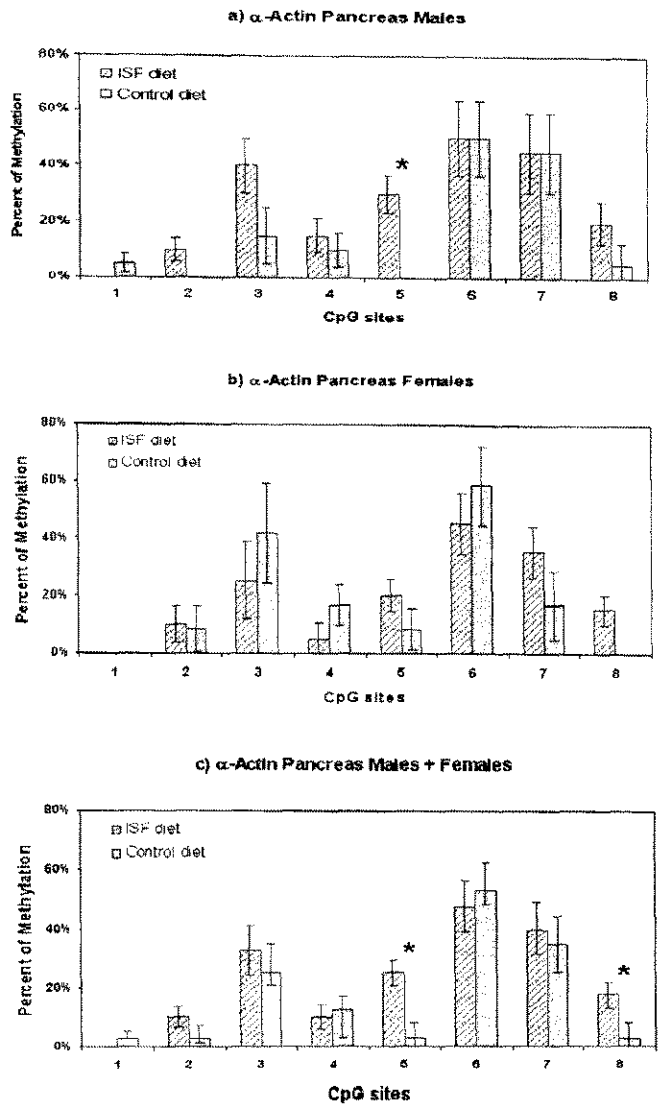


Fig. 4 – Methylation profile of the 8 CpG sites analysed for α-Actin in adult pancreas from mice born to mothers consuming control or ISF diet. Methylation values per CpG site were calculated measuring the high of methylated or unmethylated peaks in the raw data electrophenograms from direct sequencing and then the median of the methylation values in triplicate was estimated for each animal. (**a**) represents comparison between males (n = 5 for control and n = 5 for ISF); (**b**) represents comparison between females (n = 3 for control and n = 5 for ISF); (**c**) represents comparison between control and ISF groups, pooling male and female data (n = 8 for control and n = 10 for ISF). Values are expressed as the means of the methylation values ± SE. * indicates univariate differences detected with with MANOVA and considered to be significant at $P<0.05$.

Figure 5

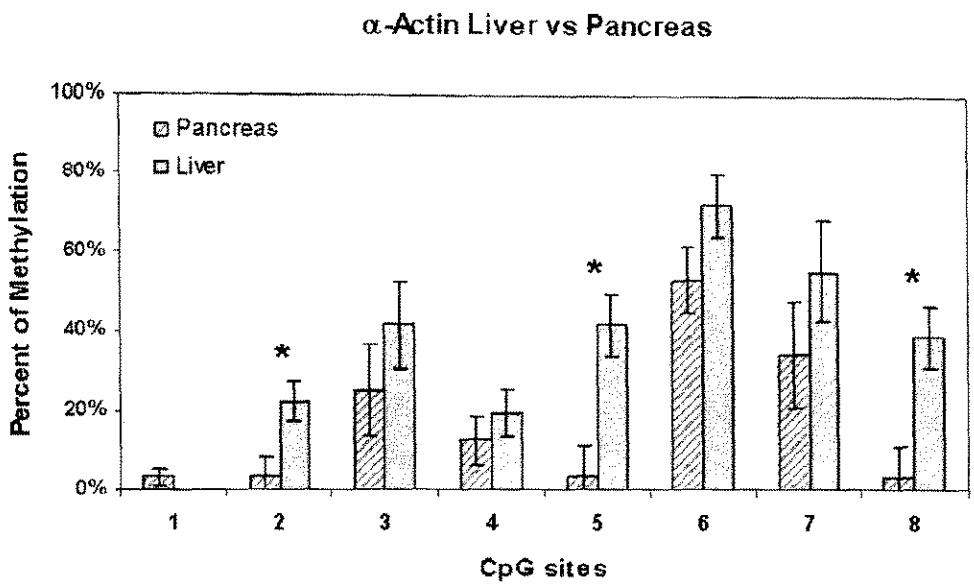


Fig. 5 – Comparison among methylation profiles for α-Actin in pancreas and liver from control mice, pooling male and female data together. Values are expressed as the means of the methylation values ± SE. * indicates univariate differences detected with with MANOVA and considered to be significant at $P<0.05$.

Figure 6) Erα CpG sites analysed

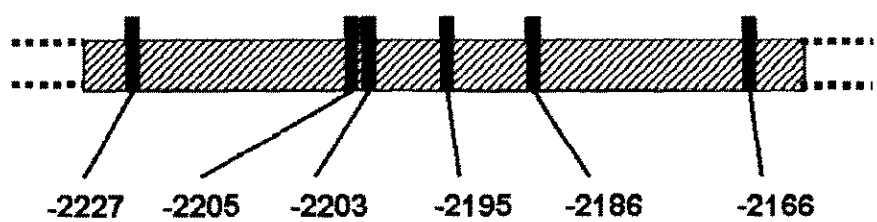


Fig. 6 – Map of the analysed region for ERα, showing the locations of CpG sites.

Figure 7

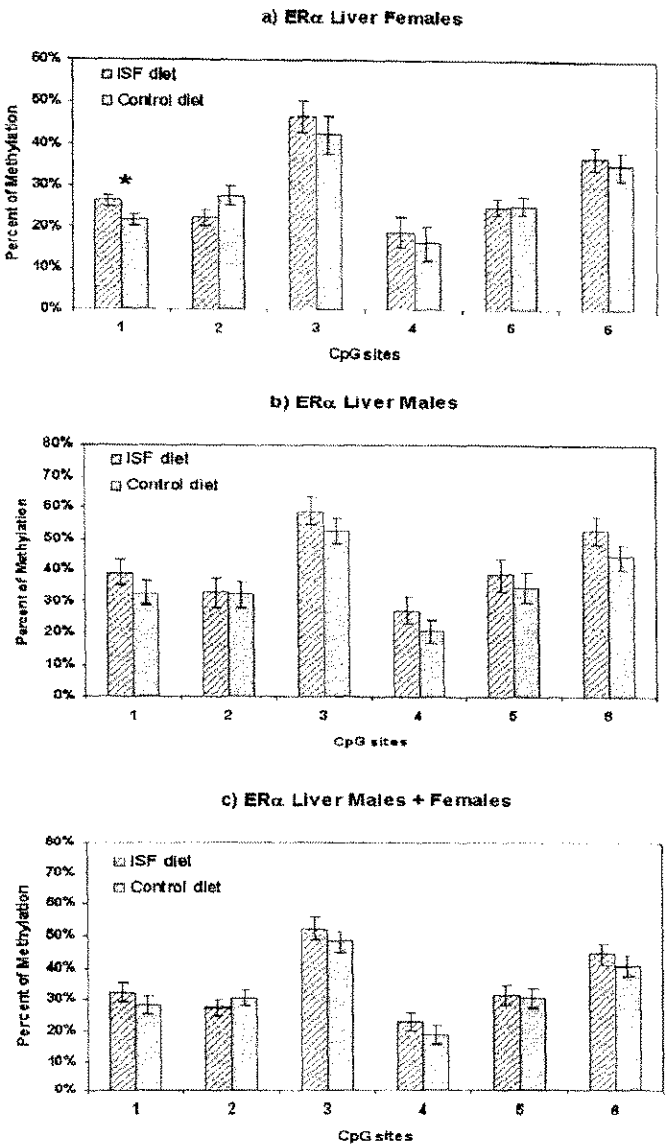


Fig. 7 – Methylation profile of the 6 CpG sites analysed for ERα in adult liver from mice born to mothers consuming control or ISF diet. Methylation values per CpG site were calculated measuring the high of methylated or unmethylated peaks in the raw data electrophenograms from direct sequencing and then the median of the methylation values in triplicate was estimated for each animal. (**a**) represents comparison between females (n = 4 for control and n = 5 for ISF); (**b**) represents comparison between males (n = 5 for control and n = 4 for ISF); (**c**) represents comparison between control and ISF groups, pooling male and female data (n = 9 for control and n = 9 for ISF). Values are expressed as the means of the methylation values ± SE. * indicates univariate differences detected with with MANOVA and considered to be significant at $P<0.05$.

The other sequence analysed was the repeated sequence LINE in blastocysts, in which 12 CpG sites were analysed. Here the analyses were performed with bisulphite PCR clonal sequencing. Due to the high number of base change detected when comparing with all the other genes analysed, we established three categories for the statistical analysis that were methylated, unmethylated and mutated. All data on methylation and base change for LINE elements, which was obtained through clonal sequencing, is shown in tables 1a and 1b. Multivariate ANOVA were performed for blastocysts LINES and no differences were found in "methylation" among blastocysts originated from mothers treated with control or ISF diet (Fig. 8a). No differences were found with the multivariate analysis for "mutation" as well. Nevertheless, CpG site # 6 and CpG site # 12 ($p<0.06$) showed CpG site specific differences in base change among blastocysts proceeding from mothers treated with control diet or ISF diet (Fig. 8b). The treatment significantly reduces base change in both sites.

Figure 8

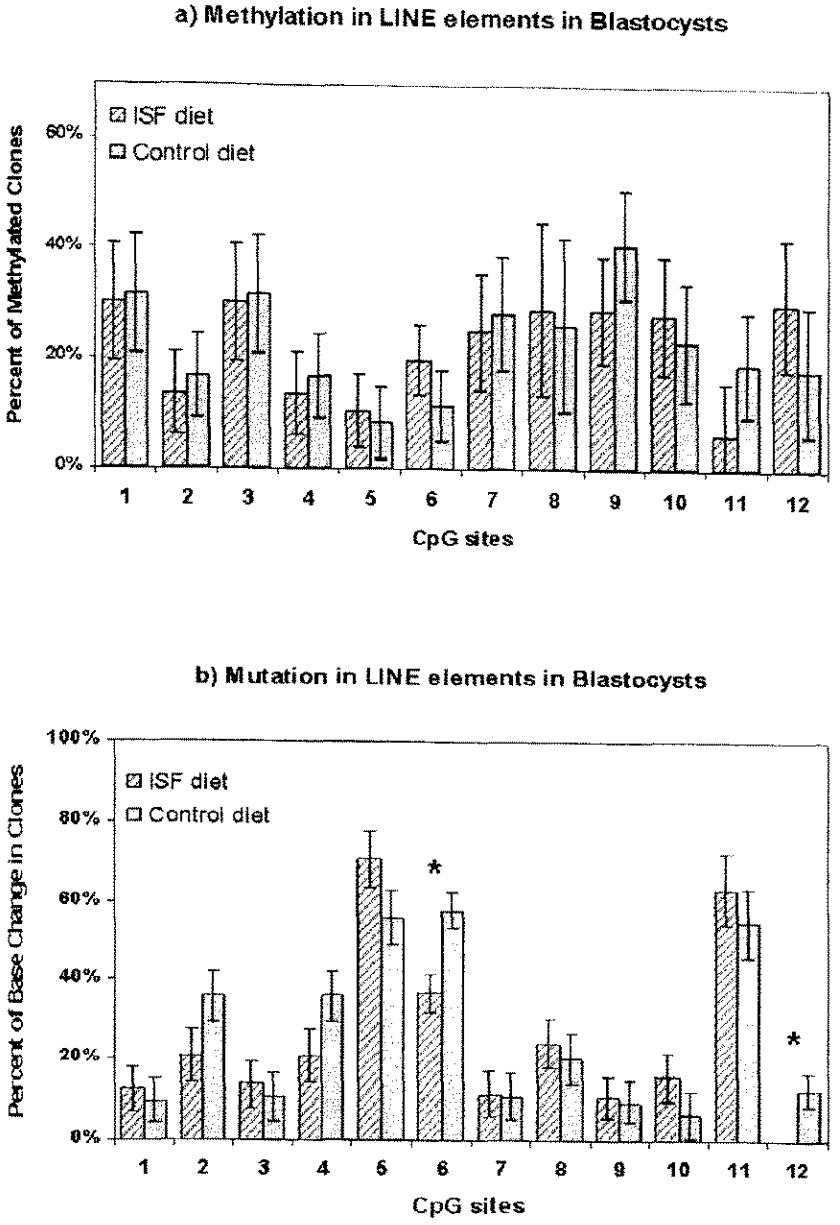


Fig. 8 – Methylation (**a**) and Mutation (**b**) profiles of the 12 CpG sites analysed for LINE elements in pre-implantation blastocysts from mothers consuming control or ISF diet. Methylation or base change values per CpG site were calculated detecting methylation or base change in peaks from electrophenograms obtained after cloning and sequencing. The percent of methylation or base change in clones was established for each pool of blastosysts from each mother. n = 4 pools of blastocysts from control mothers and n = 4 pools of blastocysts from ISF mothers. Values are expressed as the means of the methylation values ± SE. * indicates univariate differences detected with with MANOVA and considered to be significant at $P<0.05$.

TABLE 1A: Blastocysts methylation state at LINE CpG sites for samples from Control diet fed mothers

	Animal Label	CpG sites											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
	Control 1	+	-	+	-	CA	-	-	+	-	-	+	-
		+	+	+	+	AA	+	+	+	+	+	CA	+
		-	-	-	-	-	CA	CA	+	+	-	+	-
		-	CA	-	CA	+	CA	+	+	-	-	CA	CA
		-	CC/CT	-	CC/CT	CA	-	-	-	CA	CA	-	-
		+	+	+	+	-	CA	+	+	+	+	+	+
	% of Methylation	50.0	33.3	50.0	33.3	16.7	16.7	50.0	83.3	50.0	33.3	50.0	33.3
	% of Base change	0.0	33.3	0.0	33.3	50.0	50.0	16.7	0.0	16.7	16.7	33.3	16.7
	Control 2	-	CA	-	CA	-	CA	-	CA	-	-	-	CA
		CA	-	CA	-	CA	CA	-	-	-	-	-	-
		-	-	-	-	-	CA	-	-	+	-	CA	-
		-	CC/CT	-	CC/CT	CA	-	-	-	-	-	CC/CT	-
	% of Methylation	0	0	0	0	0	0	0	0	25	0	0	0
	% of Base change	25	50	25	50	50	75	0	25	0	0	50	25
	Control 6	-	-	-	-	-	CA	-	-	-	-	CA	-
		CA	-	CA	-	-	-	+	+	CA	+	+	+
		-	-	-	-	-	CA	-	-	+	CC/CT	CA	CA
		-	CC/CT	-	CC/CT	CA	CA	+	-	+	+	CC/CT	+
		-	CC/CT	-	CC/CT	CA	CA	GG	-	-	-	CC/CT	-
		+	-	+	-	CA	+	-	+	+	-	CA	-
		+	CC/CT	+	CC/CT	CA	-	+	-	+	-	CC/CT	-
		-	CC/CT	-	CC/CT	CA	-	GG	-	-	-	CC/CT	-
		+	-	+	-	CA	+	CA	CA	+	CA	+	+
		-	+	CA	+	-	CA	-	+	+	+	+	+
		+	-	+	-	CA	+	+	CA	-	+	+	CA
		-	CC/CT	-	CC/CT	CA	CA	GG	-	+	-	CC/CT	+
		+	CC/CT	+	CC/CT	-	CA	+	+	CA	+	+	+
		+	+	+	+	CA	CA	CA	-	CA	+	CT	+
		CA	-	CA	-	-	CA	-	-	-	-	CA	-
		-	-	-	-	CA	CA	-	-	-	-	CA	-
		-	-	-	-	CA	CA	-	-	-	-	-	-
		+	+	+	+	CA	-	+	CA	-	+	+	+
		+	+	+	+	CA	-	+	+	+	+	-	-
		+	-	+	-	CA	CA	CA	GG	+	+	CA	+
		+	-	+	-	CA	-	-	-	CC/CT	-	-	-
		CA	-	CA	-	CA	CA	-	CA	-	-	-	-
		-	-	-	-	CA	-	-	-	CA	+	-	-
	% of Methylation	43.5	17.4	43.5	17.4	0.0	13.0	30.4	21.7	39.1	43.5	26.1	39.1
	% of Base change	13.0	26.1	17.4	26.1	73.9	56.5	26.1	21.7	21.7	8.7	52.2	8.7
	Control 7	-	-	-	-	+	CA	+	-	+	-	CA	-
		-	-	-	-	CA	-	-	CA	-	-	CA	-
		+	CC/CT	+	CC/CT	CA	+	+	-	-	+	CC/CT	-
		-	-	-	-	-	-	-	CA	+	-	-	-
		-	CC/CT	-	CC/CT	CA	CA	-	-	+	-	CC/CT	-
		+	+	+	+	-	CA	-	-	-	-	CA	-
	% of Methylation	33.3	16.7	33.3	16.7	16.7	16.7	33.3	0	50	16.7	0	0
	% of Base change	0	33.3	0	33.3	50	50	0	33.3	0	0	83.3	0
	Methylation mean % for Control group	**31.7**	**16.8**	**31.7**	**16.8**	**8.33**	**11.6**	**28.4**	**26.3**	**41**	**23.4**	**19**	**18.1**
	Base change mean % for Control group	**9.51**	**35.7**	**10.6**	**35.7**	**56**	**57.9**	**10.7**	**20**	**9.6**	**6.34**	**54.7**	**12.6**

TABLE 1B: Blastocysts methylation state at LINE CpG sites for samples from ISF diet fed mothers

Animal Label	CpG sites											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Treatment 1	-	-	-	-	CA	CA	-	CA	-	-	-	-
	-	-	CC/CT	-	CC/CT	-	-	-	-	-	CC/CT	+
	+	+	+	+	CA	-	-	-	-	+	+	+
	-	CA	-	CA	CA	CA	-	+	+	+	CC/CT	-
	-	-	-	-	+	CA	-	+	+	+	CA	-
	-	-	-	-	-	CA	-	+	+	-	CA	-
	+	-	+	-	CA	-	-	CA	CA	CA	+	-
	CA	-	CA	-	-	CA	-	-	-	-	CA	-
	+	+	+	+	CA	CA	+	+	+	+	+	+
	-	-	-	-	-	CA	-	-	-	-	-	-
	-	CC/CT	-	CC/CT	CA	-	-	-	-	CA	-	-
	+	-	+	-	CA	-	-	+	+	+	+	+
	-	-	-	-	CA	-	CA	-	CA	CA	CA	+
	+	+	+	+	CA	+	GA	+	+	+	+	+
	CC/CT	-	CC/CT	-	CA	+	+	CA	-	-	CC/CT	+
	CA	-	CA	-	-	CA	-	-	-	-	CA	-
	-	CC/CT	-	CC/CT	CA	CA	-	-	-	-	CC/CT	-
	+	CA	+	CA	CA	CA	+	-	+	+	CC/CT	+
	CA	-	CA	-	CA	-	-	-	+	+	-	+
	-	-	-	-	-	-	CA	-	-	-	CC/CT	-
	-	-	-	-	-	+	-	+	+	+	CA	+
	+	+	+	+	CA	-	-	-	+	-	-	+
	+	-	+	-	+	CA	+	-	+	+	CA	+
	+	+	+	+	CA	-	-	+	+	+	+	+
% of Methylation	37.5	20.8	37.5	20.8	8.3	12.5	16.7	33.3	50.0	45.8	25.0	54.2
% of Base change	16.7	16.7	20.8	16.7	66.7	45.8	12.5	12.5	8.3	12.5	54.2	0.0
Treatment 2	+	-	+	-	CA	CA	-	+	-	-	CC/CT	-
	-	-	-	-	-	+	+	+	+	-	CA	-
	-	-	-	-	CA	CA	-	-	-	-	-	-
	+	-	+	-	CA	+	+	CA	-	+	CA	+
	-	-	-	-	CA	-	CC/CT	CA	-	-	-	-
	CA	-	CA	-	CA	-	-	+	CC/CT	-	-	-
% of Methylation	33.3	0.0	33.3	0.0	0.0	33.3	33.3	50.0	16.7	16.7	0.0	16.7
% of Base change	16.7	0.0	16.7	0.0	83.3	33.3	16.7	33.3	16.7	0.0	50.0	0.0
Treatment 3	-	CA	-	CA	CA	-	-	-	CC/CT	-	CA	+
	+	+	+	+	+	CA	+	-	+	+	CA	+
	CC/CT	-	CC/CT	-	-	-	-	CA	-	-	-	-
	+	+	+	+	CA	+	CA	+	+	+	CC/CT	-
	-	CA	-	CA	+	CA	+	+	+	CA	CA	+
	+	-	+	-	CA	+	+	CA	-	+	CA	-
% of Methylation	50.0	33.3	50.0	33.3	33.3	33.3	50.0	33.3	50.0	50.0	0.0	50.0
% of Base change	16.7	33.3	16.7	33.3	50.0	33.3	16.7	33.3	16.7	16.7	83.3	0.0
Treatment 4	-	-	-	-	CA	-	-	-	-	-	CA	-
	-	-	-	-	CA	-	-	CA	-	GG	-	-
	-	CC/CT	-	CC/CT	CA	-	-	-	-	-	CA	-
	-	-	-	-	CA	-	-	-	-	-	-	-
	-	-	-	-	-	CA	-	-	-	GG	CA	-
	-	CC/CT	-	CC/CT	CA	CA	-	-	-	-	CC/CT	-
% of Methylation	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
% of Base change	0.0	33.3	0.0	33.3	83.3	33.3	0.0	16.7	0.0	33.3	66.7	0.0
Methylation mean % for ISF group	**30.2**	**13.5**	**30.2**	**13.5**	**10.4**	**19.8**	**25**	**29.2**	**29.2**	**28.1**	**6.25**	**30.2**
Base change mean % for ISF group	**12.5**	**20.8**	**13.5**	**20.8**	**70.8**	**36.5**	**11.5**	**24**	**10.4**	**15.6**	**63.5**	**0**

DISCUSSION

The analysis of methylation in promoter regions of specific genes as ERα and α-Actin revealed that the consumption of high amounts of dietary phytoestrogens (in the present case represented by a diet containing 2% of a soy extract of isoflavones) by mice mothers lead to punctual but not global changes in CpG sites methylation in the offspring. For α-Actin, it is interesting to notice that there is a natural difference (seen in controls) among males and females in CpG sites # 4 and # 7, with males tending to have decreased levels of methylation in these sites in regard to females. Nevertheless, the treatment with dietary isoflavones seems to cause a suppression of such gender differences, increasing the levels of methylation in both # 4 and 7 CpG sites in females. In addition to those differences observed in liver, interesting results appeared for α-Actin in pancreas as well. A significant increase in methylation was detected for site # 5 in males from ISF group with respect to controls. Although this change is not observed in females, analysis of male and female data pooled together showed increased methylation in response to ISF treatment in two CpG sites, # 5 and # 8. In principle, this seems to be an inconsistent finding; however, it can be explained by the number of individuals in each comparison. Pooling male and female data increased the number of individuals and leads the MANOVA to show differences that were hidden when performing the analysis with fewer data. Thus, according to what is shown in fig. 4 a, b and c, the difference in pooled data observed for site # 5 is due to a male difference; nevertheless, the difference in pooled data observed for site # 8 is due to pre-existing independent differences for each gender, which shows up when the statistical test increases its power in response to the higher number of individuals in the analysis. We also found one site specific difference produced by the ISF treatment in the site # 1 in Erα.

The differences in methylation seen for both genes analysed does not show a defined global pattern regarding the direction of the changes. In some sites methylation is increased by the treatment but in others is decreased. However, there are specific sites that seem to be specific targets for specific stimulus that could interact with those previously mentioned factors as RNA factors, histone methylation and chromatin remodelling enzymes in order to establish a methylation pattern. The direction of the changes must be defined by the interaction among these factors and not solely by the stimulus, although the stimulus is certainly producing influences leading to a specific change. Moreover, such specific methylation change is not at random, but consistent with the stimulus that triggered that change, producing a pattern response. This is interesting from a evolutionary point of view because indicates that certain class of environmental compounds are able to cross the uterus barrier in mammals and induce biased modifications in methylation patterns in embryos.

Another interesting finding is one that corroborates the tissue specific differences in methylation of the mice α-Actin promoter seen in Warnecke & Clark (1999). In the present study, we found for α-Actin that pancreas shows a different methylation profile as liver or as all the other tissues analysed in Warnecke & Clark (1999) such as heart, skeletal muscle and kidney. Tissue specific differences in methylation profiles were also detected for ERα, which show absence of methylation in pancreas, contrasting with a well defined methylation profile seen in liver.

The question arising at this point is: when and how during development these differences in methylation are established? Other authors have also described tissue specific methylation.

Song et al. (2005) identified 150 spots produced by restriction landmark genomic scanning in the mice genome, which presented methylation in some tissues but not in others. One alternative for explain such differences in methylation among differentiated tissues is that important events of methylation could be occurring when organs are been formed in the embryo at the time of organogenesis, in addition to those well known events occurring in pre-implantation (Reik et al., 2001) and post-implantation blastocysts (Constância et al., 1998) or during gastrulation (Mac Phee, 1998). Nevertheless, differences in methylation patterns in adult tissues can also be due to active demethylation. In this sense, Bruniquel & Schwartz (2003) reported that in a small region of the interleukine gene, demethylation takes place in T lymphocytes following activation and that such demethylated state is maintained thereafter.

In addition to the effects of maternal consumption of phytoestrogens on methylation of specific genes, we also analysed eventual changes in methylation or occurrence of mutations in the repeated sequence LINE in blastocysts from mothers subjected to the isoflavone treatment. The results on LINE elements show that although specific changes in methylation are seen for the other sequences analysed, global methylation patterns are not changed in blastocysts due to the maternal treatment with isoflavones. This recall the importance of analysing methylation data with a CpG site sensitive method such as bisulphite sequencing, which permits to detect changes in methylation that otherwise can not be detected.

Also interesting is the finding of reduced base change in two CpG sites (# 6 and 12) in LINE elements. This fact could also be related with methylation. It is known that the

methylated form of CpG has 12-fold higher mutation rate to TpG and CpA (Sved & Bird, 1990). In the present study, the reduction of base change in two CpG sites in LINE elements from blastocysts from mothers consuming ISF diet could be related with the above mentioned pattern, given that in both sites the reduced mutation is to CpA in the ISF group. This could means that what is in fact occurring is a reduction in methylation in those CpG sites prior to mutation, leading latter to a decreased rate of mutation to CpA. Although this is only a speculation, it could be a feasible event.

The goal of the present study was to determine if environmental signals as phytoestrogens could elicit changes in methylation patterns during the development and not to evaluate how these differences in methylation found could alter the pattern of expression of the involved genes. Nevertheless, the present study also opens questions regard the effect of the CpG site-specific changes seen here on the expression of ERα and α-Actin. It is interesting to extrapolate the effect on methylation seen in these genes to the whole genome. Several other punctual CpG sites in genes or regions must be affected due to an early influence with isoflavones or other environmental signal capable of crossing the uterus barrier and of affecting the developing embryo. This could have consequences on gene expression and finally, on the phenotype itself. This emphasises the importance of natural compounds on influencing an epigenetically regulated mechanism involved in gene regulation and transgenerational perpetuation of characters, such as DNA methylation. Thus, prenatal development in mammals is a sensitive process that strongly depends on maternal diet and furthermore, maternal consumption of compounds as phytoestrogens (and maybe many

others) may influence and bias the process of establishment of methylation patterns in mammalian embryos.

REFERENCES

Anway, M. A., Cupp, A. S. and Uzumcu, M. & Skinner, M K. (2005) *Science* **308**, 1466-1467.

Beaver, B. V., Reed, W., Leary, S., McKiernan, B., Bain, F., Bennett, B. T., Pascoe, P., Shull, E., Cork, L. C., Francis-Floyd, Amass, K. D., R., Johnson, R., Schmidt, R. H., Underwood, W., Thornton, G. W. and Khon, B (2001) *J. Am. Vet. Med. Assoc.* **218**, 669-696.

Bruniquel, D. & Schwartz, R. H. (2003) *Nat. Immunol.* **4**, 235-240.

Chen, Z. X. & Riggs, A. D. (2005) *Biochem. Cell. Biol.* **83**, 438-48.

Clark, S. J. & Frommer, M (1997) in *Laboratory methods for the detection of mutations and polymorphisms in DNA* (G. R. Taylor, ed., CRC Press, Boca Ratón, FL), pp. 151-162.

Clark, S. J., Harrison, J., Paul, C. L. & Frommer, M. (1994) *Nucleic Acids Res.* **22**, 2990-2997.

Constância, M., Pickard, B., Kesley, G., & Reik, W. (1998) *Genome Res.* **8**, 881–900.

Day, J., Bauer, A., desBordes, C., Zhuang, Y., Kim, B., Newton, L., Nehra, V., Forsee, K., MacDonald, R., Besch-Williford, C., Huang, T. and Lubahn, D. (2002) *J. Nutr.* **132** (supplement), 2419S-2423S.

Guerrero-Bosagna, C. Sabat, P. & Valladares, L. (2005) *Evol. Dev.* 7, 341-350.

Kafri, T., Ariel, M., Brandeis, M., Shemer, R., Urven, L., McCarrey, J., Cedar, H. and Razin, A. (1992) *Genes Dev.* **6**, 705-714.

Khosla, S., Dean, W., Brown, D., Reik, W. and Feil, R. (2001) *Biol. Reprod.* **64**, 918-926.

Kos, M., O'Brien, S., Flouriot, G., Gannon, F. (2000) *FEBS Lett.* **477**, 15-20.

Li, E (2002) *Nat. Rev. Genet.* **3**, 662-673.

Li, S., Washburn, K., Moore, R., Uno, T., Teng, C., Newbold, R., McLachlan, J. and Negishi, M. (1997) *Cancer Res.* **57**, 4356-4359.

Lyn-Cook, B. D., Blann, E., Payne, P. W., Bo, J., Sheehan D. and Medlock, K. (1995) *Proc. Soc. Exp. Biol. Med.* **208**, 116-119.

Mac Phee, D. (1998) *Mutat. Res.* **400**, 369-379.

Monk, M., Boubelik, M. and Lehnert, S. (1987) *Development* **99**, 371-382.

Reik, W., Dean, W. and Walter, J. (2001) *Science* **293**, 1089-1092.

Sved, J. and Bird, A. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 4692-4696.

Waalkes, M. P., Liu, J., Chen, H., Xie, Y., Achanzar, W. E., Zhou, Y. S., Cheng, M. L. & Diwan, B. A. (2004) *Nat Immunol.* **4**, 235-240.

Warnecke, P. & Clark, S. (1999) *Mol. Cell. Biol.* **19**, 164-172.

Warnecke, P. M., Stirzaker, C., Song, J., Grunau, C., Melki, J. R. & Clark, S. J. (2002) *Methods* **27**, 101-107.

Wu, Q., Ohsako, S., Ishimura, R., Suzuki, J.S. and Tohyama, C. (2004) *Biol. Reprod.* **70**, 1790-1797.

Zuccotti, M. & Monk, M (1997) *Nat. Genet.* **9**, 316-320.

A

FINAL CONCLUSIONS AND FUTURE DIRECTIONS –

Developmental epigenetics is a broad phenomenon, which has initially been described by Waddington to refer to "the branch of biology which studies the causal interactions between genes and their products which bring phenotypes into being". Nowadays, however, the study of epigenetic regulation of development has been sharpened as a result of recent work on molecular mechanisms of gene expression and developmental biology (Jablonka et al., 2002). Therefore, at the present, epigenetics is a well accepted phenomenon by the scientific community, mainly due to the recent discoveries in the field of the molecular biology, namely chromatin condensation, histone acetylation and deacetylation or DNA methylation, which are all well identified processes.

Nevertheless, the searching for functionality in every studied structure in organisms is also a fact in present biology. Often, researchers are attributing nature with needs and purposes that derive from our human practices (Bunnel, 2000). In this sense, epigenetic studies reporting the possibility of new phenotypes being induced by environmental agents is a strong argument to contradict the widespread use of the purpose, which is inserted in the adaptationistic paradigm in current evolutionary biology. When some agent induces a new phenotypic conformation, the form in which it will arise is unknown. The only certain fact is that there is some constrains either at mechanic, physiological and/or molecular levels that limits the possibility of certain changes. But once the organism has overcome such barriers and some changes could be achieved, the following step is the survivor of the organism as a whole. And is in the relation between the organism and the environment that the emergent interactions will or not enable the organism to live with the achieved modifications. The epigenetic changes produced by environmental stimuli can be achieved does not matter how the organism will be using such changes regarding to its

surrounding environment or to the initial stimuli (Guerrero-Bosagna et al., 2005). In spite of this, the organismal responses to such stimuli certainly produce patterned phenotypes. Each stimulus can trigger one or more particular responses, but that should be always similar if the conditions of the stimuli upon the organism are maintained. In Waddington's (1957) words, "we must realise that there should be a specific homeorhetic cross-section corresponding to each different kind of influence which affects the system" (an organismal system). In other words, this would correspond to phenotypical bias or patterns, induced by the environment.

In this sense, a general conclusion of the experimental work performed in this thesis is that certain kind of environmental compounds when acting at well defined windows of time in mammals are able to induce pattern responses at several levels in the organisms, among them, physiological, morphological or molecular. Such pattern responses produce new phenotypes that are not responding to any adaptationistic goal imposed by the environment. The organism only responds with their possibilities as living structures they are.

Summarizing the results obtained in the present work, subjecting an experimental population of mice to a high consumption of phytoestrogens leads to alterations in morphometric and life-history characters in the offspring, which are: changes in adult gene specific methylation patterns, reduced adult weight (males) and advancement of sexual maturation (females). All those changes are not necessarily responding as adaptations to environmental challenges, rather, they are structurally responding to specific stimuli at a particular time window during ontogeny that permits such a response.

As future questions to investigate that emerged from the experimental work performed in this thesis, I would like to point out the following issues:

- Intrauterine catecholestrogens: after developing the technique to measure catecholestrogens in biological fluids, the main question arising is whether nutritional phytoestrogens have an association with the intrauterine secretion of 2-OH-Estradiol or 4-OH-Estradiol during pre-implantation development.
- An interesting approach to evaluate the possible role of catecholestrogens in the process of DNA methylation in the early embryo would be to culture blastocysts in vitro with variable levels of cathecholestrogens and observe the consequences in methylation patterns either in pre-implantation blastocysts or adults.
- Male offspring reduction in weight due to the ISF treatment: are such differences due to increased levels of circulating testosterone in these animals?
- In addition the two gene specific methylation changes due to the maternal treatment with phytoestrogens observed in the present study, would be interesting to scanning for eventual changes in the whole genome with techniques as Amplification of Intermethylated Sites (AIMS) (Frigola et al., 2002) or PCR-based methylation-sensitive HpaII analysis (Anway et al., 2005).
- Regarding the sex ratio differences observed, the question arising is if the increased female proportion is in fact due to a methylation-produced silencing in the sex determining region Y (SRY) at a very precise time during early development (between days 10.5 and 12.5 post fertilization) in males, which would generate increased number of females.

- An interesting populational approach would be to know how the changes observed in the present study in female sexual maturation and litter sex proportion will reflect changes in the structure of a population in parameters as age and sex composition, or in the percent of reproductive individuals. Furthermore, how could such changes influence population dynamics.

In addition to those specific future aims, in a broad sense my future lines of research will focus on the following issues that in my opinion are not well explored in current evolutionary biology: i) to identify environmental compounds capable to produce epigenetic modification of the DNA, ii) to detect the window of time along the ontogeny of different organisms, when epigenetic modification of DNA is more susceptible to be produced and iii) to study the consequences of epigenetic modification of DNA in populational parameters. The precise establishment of the limits of the window of time when such DNA changes are more susceptible to occur is an exciting challenge for epigenetic studies, especially in organisms with sexual reproduction.

REFERENCES

Anway, M. A., Cupp, A. S. and Uzumcu, M. & Skinner, M K. 2005. Epigenetic transgenerational actions of endocrine disruptors and male fertility. *Science,* 308, 1466-1467.

Bunnel, P. 2000. Attributing nature with justifications. *Syst. Res.*, 17, 469-480.

Frigola J., Ribas M., Risques R. A. and Peinado M. A. 2002. Methylome profiling of cancer cells by amplification of inter-methylated sites (AIMS). Nucleic Acids Res., 30, e28.

Guerrero-Bosagna,C., Sabat, P. & L. Valladares. 2005. Environmental signaling and evolutionary change: can exposure of pregnant mammals to environmental estrogens lead to epigenetically-induced evolutionary changes in embryos? *Evolution & Development*, 7: 341-350.

Jablonka, E., Matzke, M., Thieffry, D. & Van Speybroeck, L. 2002. The genome in context: biologists and philosophers on epigenetics. *BioEssays,* 24: 392-394.

Waddington, C.H. 1957. The strategy of the genes. Printed by J.W. Arrowsmith LTD, London.

VI

Separation and quantification of the catecholestrogens 4-OH-Estradiol and 2-OH-Estradiol by HPLC: an improved procedure for measurement of potential cancer biomarkers

Carlos Guerrero-Bosagna,*[ab] Fernando Garrido[a] and Luis Valladares[a]

[a] Laboratorio de Hormonas y Receptores. Instituto de Nutrición y Tecnología de los Alimentos (INTA), Universidad de Chile, Av. Macul 5540, Macul, Santiago, Chile. Fax: +56-2-223 4030; Tel: +56-2-978 1435

[b] Laboratorio de Ecofisiología Animal, Facultad de Ciencias, Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago, Chile. Fax: +56-2-272 7363; Tel: +56-2-978 7232

*Author for correspondence, E-mail: catelo@inta.cl

Key Words: Catecholestrogens; HPLC; 4-hydroxyestradiol; 2-hydroxyestradiol; cancer markers.

Abstract

Catecholestrogens (CEs) are compounds derived from the estrogen metabolism. CEs have been identified in several biological materials and implicated with oncogenic activity. Determination and separation of CEs from biological fluids is commonly carried out by derivatization in liquid chromatography followed by mass spectrophotometry or gas chromatography. However, these methods allow less resolutive identification of such metabolites respect to HPLC. Furthermore, the CEs 2-hydroxyestradiol and 4-hydroxyestradiol have never been separated in a resolutive manner. We describe a simplified method to separate and quantify them through HPLC with electrochemical detection, using a monolithic instead of a particulate column, which reduces column backpressure.

1. Introduction

Catecholestrogens (CEs) are compounds derived from estrogen metabolism and produced through hydroxylation at the position C2 or C4 of the estrogen molecule, leading to the formation of 2-OH-estradiol (2-OH-E2) and 4-OH-estradiol (4-OH-E2), respectively.[1,2] CEs have been identified in several biological materials as urine,[3] kidney,[4] brain,[5] liver and uterus[6] of rodents. Recently, CEs have been associated with oncogenic processes, after demonstration that its production by hamster kidney, in particular of 4-OH-E2, is related to carcinogenic activity.[4] CEs are usually inactivated by conjugation reactions, such as methylation, glucoronidation or sulfation.[2,7] If production of these conjugates is insufficient, CEs may be oxidized to CE quinones or semi-quinones.[2,8] Such derivates can be reduced or conjugated by other compounds, however, if such processes are not sufficient, the non-conjugated quinone and/or semi-quinones will form stable adducts capable of binding to DNA[2]. This can lead to the formation of potentially carcinogenic stable or depurinating DNA adducts.[8] CEs also participate in other physiological processes, such as embryo implantation. In mice, CEs mediate the activation of blastocysts prior to implantation.[9]

In humans, there have been attempts to use the steroid excretion profiles of both classical and catechol estrogens in the detection of hormonal and non-hormonal tumours.[10] Goodman *et al.*[7] suggested that CE formation is associated with ovarian carcinogenesis. Furthermore, Liehr and Ricci[11] have found an elevated ratio of 4-OH-E2 formation with respect to 2-OH-E2 in neoplastic mammary tissue, which would serve as a useful marker of benign or malignant breast tumors.

Despite the increasing knowledge on the effects of CEs and their role in cancer formation, research has been hampered because CEs are unstable and methods for detecting them are labour intensive.[12] To better understand the relationship between CEs and cancer, or among CEs and embryo implantation in mammals, a sensitive and practical method is needed in order to determine and quantify CEs presence in those biological fluids known to contain them, or in microsomal mediated production of CEs.

The first techniques to separate and quantify CEs relied on HPLC assays.[3,6,13,14] Some recent work, however, has employed derivatization in liquid chromatography followed by mass spectrophotometry, which permits identification of CEs but not their quantification.[5] Gas chromatography has also been used for an effective separation.[15,16] However, in order to analyse steroid metabolic patterns in vitro, HPLC is preferable because of its selectivity, specificity and sensitivity[10]. In spite of this, resolution among 2-OH-E2 and 4-OH-E2 separation through liquid chromatography is still critical and the development of better conditions in order to improve its separation is needed.

In the present work we describe a method to quantify and separate 2-OH-E2 and 4-OH-E2 by HPLC with electrochemical detection, allowing unambiguous separation of such CEs. For it, we used a monolithic column, which significantly reduced column backpressure as compared to particulate columns. Such pressure reduction allows working with increased flow rates, which made the separation of these CEs possible. In the analysis we document chromatografic parameters such as retention times, selectivity, resolution, limit of detection and limit of quantification.

2. Experimental

2.1 Chemicals

Standards of 4-OH-E2 and 2-OH-E2 were supplied by Sigma. Citric acid was from Winkler Ltda, ammonium acetate from Merck and glacial acetic acid from J. K. Baker Chemical Co. All solvents used (ethanol, methanol and acetonitrile) were HPLC grade and supplied by Omnisolv, EM Science.

2.2 Liquid chromatography

The HPLC system consisted of a Bioanalytical Systems Inc. (BAS) CC-5 cabinet used as a Faraday cage. It also accommodated other components of a liquid chromatography system such as the column,

column heater, injection valve, BAS cross-flow thin layer cell with stainless steel auxiliary electrode, glassy carbon working electrode and a RE-6 downstream reference electrode.

The controller used was an amperometric detector ClinRep L-3500A (Merck-Recipe) connecting a system Merck-Hitachi HPLC- Manager D-6000, intelligent pump L-6200A and computer. We used Chromatography Data Station Software for Model D-6000 HPLC Manager (Hitachi). The injection device was a Rheodyne, model 7125i (Cotati, CA, USA) with a 20 µl sample loop. The column was the Merck Chromolit Performance RP-18e 100-4.6 (100 mm x 4.6 mm).

The mobile phase was a mixture of 21% acetronitrile and 79% buffer citric acid (75 mM)-ammonium acetate (25 mM). The mobile phase was run into the column at a flow rate of 1.5 ml min^{-1} and a constant temperature of 40° C. The potential of the electrochemical detector was set at 0.6 V versus reference electrode.

2.3 Calibration Curve

Stock standard solutions of 4-OH-E2 and 2-OH-E2 were prepared at a concentration of 0.1 mg ml^{-1} in 1 M acetic acid diluted in ethanol. From these, stock solutions of 0.2 µg ml^{-1}, diluted in 1 M acetic acid in ethanol, were prepared for both CEs. All stock solutions were frozen at –20 °C protected from light exposure and under nitrogen, in order to avoid degradation and oxidation. Further, working standard solutions of seven different concentrations were prepared to construct the calibration curve. These dilutions were in methanol/H2O (1:1). For 4-OH-E2 we used the following concentrations: 5, 20, 40, 60, 100, 150 and 200 ng ml^{-1}; for the 2-OH-E2 we used the following concentrations 2, 10, 20, 30, 50, 70 and 100 ng m^{-1}.

3. Results and Discussion

3.1 Linearity

Linearity was verified within the range of standard concentrations used for each 2-OH-E2 and 4-OH-E2. Calibration curves were constructed by plotting peak area (expressed in mV x min) versus concentration of the injected standard. The parameters obtained from the calibration curves for both analyzed compounds are shown in Table 1.

Table 1

[a] $y = Ax + B$; y = concentration of the injected sample (ng ml^{-1}); x = peak area (mV min^{-1}); [b]degrees of freedom = 1, 40; [c]degrees of freedom = 1, 35.

Compound	Slope (A^a) (X 10^4)	Intercept (B^a) (X 10^4)	Regresion coefficient (r^2)	F (X 10^5)	p	Syx (X 10^5)
2-OH-E2	15.4	-17.9	0.9875	3.2[b]	<< 0.01	5.8
4-OH-E2	5.4	-6.7	0.9904	3.6[c]	<< 0.01	3.6

Parameters of linear regression for 2- and 4-OH-E2 calibration curves and ANOVA test.

3.2 Peak identification, separation and resolution

Peaks were identified based on retention times (t_R), which were calculated for each CE by estimating the mean of t_R measurents for the corresponding calibration curve. The t_R values for both CEs are shown in Table 2. According to our data, we obtained a separation of 2.15 min between 2- and 4-OH-E2. Such separation can unambiguously identify these CEs in biological fluids. Additionally, we calculated resolution (Rs) in a mixture of standards of both CEs with concentrations producing band pairs with an area ratio of 1:1 (Fig. 1). Resolution was estimated as $Rs=2\ (t_2 - t_1)\ (W_1 + W_2)^{-1}$,[17] where t_1 and t_2 are the retention times of both adjacent bands and W_1 and W_2 are the corresponding baseline bandwidths. Using this equation our estimate of Rs is 1.75.

We also calculated selectivity (α), which is expressed as the ratio between the values of k for each adjacent peak (k_2/k_1). The k factor is equal to $(t_R - t_0)/t_0$ (t_R retention time; t_0 column dead time). For α we obtained a value of 1.115.

Table 2

Retention times and standard deviations for the 2- and 4-OH-E2 catecholestrogens.

Compound	Retention time (min)	S. D.
2-OH-E2	14.39	1.05
4-OH-E2	12.24	0.98

[a]n = 40; [b]n = 35.

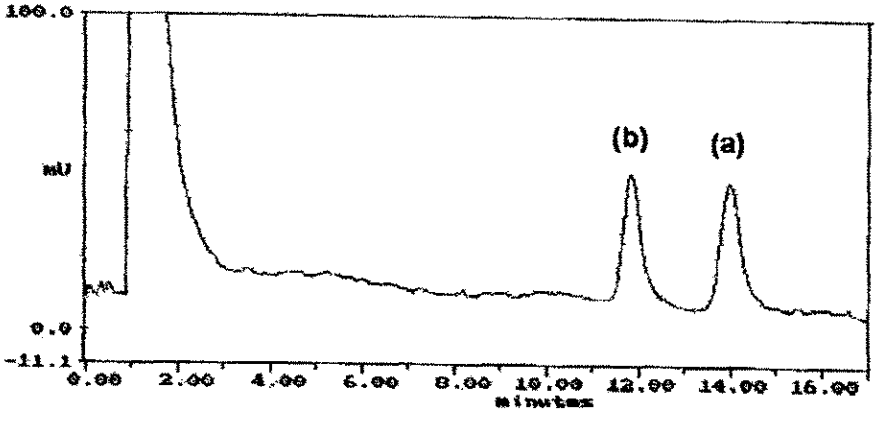


Fig. 1. Separation between (a) 2-OH-E2 and (b) 4-OH-E2 through HPLC. Injection (20 µl) of a solution of mixed standards of both catecholestrogens at the concentration of 10 ng ml^{-1} for 2-OH-E2 and of 20 ng ml^{-1} for 4-OH-E2.

The limit of detection (LOD) is defined as the smallest concentration of the analyte that can be detected reliably, and the limit of quantitation (LOQ) is the concentration that can be quantitated reliably with a specified level of accuracy and precision.[16] We estimated both the LOD and LOQ for our method. For the 2-OH-E2, the estimated LOQ and LOD were 2 and 0.6 ng ml^{-1}, respectively. For the 4-OH-E2, LOQ and LOD values were 5 and 1.5 ng ml^{-1}, respectively.

4. Conclusion

We report a simple method for the quantification and resolutive separation of the CEs 2- and 4-OH-E2. The limit of quantification of our system was 2 ng ml^{-1} for the 2-OH-E2 and 5 ng ml^{-1} for the 4-OH-E2, with a separation of 2.15 min between them. With these parameters, both CEs can be identified and separately quantified in biological materials.

The main significance of the estimation of these compounds is the relationship between their production and the association with oncogenic process. Previous work has reported that differences between 2-OH-E2 and 4-OH-E2 production could be useful as a marker of breast tumors[11]. This technique may enable these differences between such CEs to be detected and to serve as a marker in cancer research.

Acknowledgements

The authors thank Dr. Mauricio Canals for helping in the statistical analysis of data and Renée Hill and Bradley Bakken for linguistic revision. We also appreciate founding by projects Fondecyt 1010647 to Pablo Sabat and 1030309 to Luis Valladares. Carlos Guerrero-Bosagna is grateful to CONICYT and also INTA and Nestlé Chile S.A. for doctoral grants.

References

[1] P. Ball and R. Knupen, Acta Endocrinol. 93, Suppl. 232 (1980) 1.

[2] E. L. Cavalieri, D. E. Stack, P. D. Devanesan, R. Todoroviv, I. Dwivedy, S. Higginbotham, S. L. Johansson, K. D. Patil, M .L. Gross, J. K. Gooden, R. Ramanathan, R. L. Cerny and E. G. Rogan, Proc. Natl. Acad. Sci. USA 94 (1997) 10937.

[3] K. Shimada, T. Tanaka, T. Nambara, J. Chromatogr., 223 (1981) 33.

[4] B. T. Zhu, D. Roy, and J. G. Liehr, Endocrinology 132 (1993) 577.

[5] K. Mitamura and K. Shimada, Chromatography 22 (2001) 11.

[6] C. Bunyagidj and J. McLachlan, J. Steroid. Biochem. 31 (1988) 795.

[7] M. T. Goodman, K. McDuffie, L. N. Kolonel, K. Terada, T. A. Donlon, R. W. Lynne, C. Guo and L. Le Marchand, Cancer Epidemiol. Biomarkers. Prev. 10 (2001) 209.

[8] A. A. Adjei and R. M. Weinshilboum, Biochem. Biophys. Res. Com. 292 (2002) 402.

[9] B. Paria, H. Lim, X. Wang, J. Liehr, S. Das and S. Dey, Endocrinology 139 (1998) 5235.

[10] L. Castagneta, M. Granata, M. Lo Castro, G. D'Agostino, F. Mitchell, and M. J. O'Hare, Ann. N. Y. Acad. Sci. 464 (1986) 316.

[11] J. Liehr and M. Ricci. Proc. Natl. Acad. Sci. USA 93 (1996) 3294.

[12] C. De Crée, P. Ball, B. Seidlitz, G. Van Kranenburg, P. Geurten and H. Keizer, J. Appl. Physiol. 82 (1997) 364.

[13] K. Shimada, T. Tanaka, T. Nambara, J. Chromatogr. 178 (1979) 350.

[14] Catecholestrogens, Metabolites and Adducts. Application Note by ESA Inc., 22 Alpha Road Chelmsford, MA 01824-4171, U.S.A.

[15] L. Castagneta, M. Granata, P. Arcuri, L. Polito, F. Rosati and G. Cartón, Steroids 57 (1992) 437.

[16] K. D. Pinella, B. K. Cranmer, J. D. Tessari, G. D. Cosma, and D. N. Rao Veeramachaneni, J. Chromat. B 758 (2001) 145.

[17] L. Snyder, J. Kirkland and J. Glajch, in Practical HPLC method development, John Wiley & sons, Inc (Eds.), Printed in USA, 1997, p. 765.