学校编码：10384　　分类号＿＿＿密级＿＿＿
学号：21620111152482　　UDC＿＿＿

厦门大学

硕 士 学 位 论 文

锌与双酚 A 胁迫下福建牡蛎性腺差异表达蛋白的研究

Different proteomics profiling of *Crassostrea angulata* gonad exposed to Zn and bisphenol-A

张庆鸿

指导教师姓名：　黄河清 教授
　　柯才焕 教授

专　业　名　称：生物化学与分子生物学

论文提交日期：2014 年 5 月

论文答辩时间：2014 年 5 月

学位授予日期：2014 年 6 月

答辩委员会主席：＿＿＿＿＿＿

评　　阅　　人：＿＿＿＿＿＿

2014 年 5 月

厦门大学学位论文原创性声明

本人呈交的学位论文是本人在导师指导下,独立完成的研究成果。本人在论文写作中参考其他个人或集体已经发表的研究成果，均在文中以适当方式明确标明，并符合法律规范和《厦门大学研究生学术活动规范（试行）》。

另外，该学位论文为（　　）课题（组）的研究成果，获得（　　）课题（组）经费或实验室的资助，在（　　）实验室完成。（请在以上括号内填写课题或课题组负责人或实验室名称，未有此项声明内容的，可以不作特别声明。）

声明人（签名）：

年　　月　　日

厦门大学学位论文著作权使用声明

本人同意厦门大学根据《中华人民共和国学位条例暂行实施办法》等规定保留和使用此学位论文，并向主管部门或其指定机构送交学位论文（包括纸质版和电子版），允许学位论文进入厦门大学图书馆及其数据库被查阅、借阅。本人同意厦门大学将学位论文加入全国博士、硕士学位论文共建单位数据库进行检索，将学位论文的标题和摘要汇编出版，采用影印、缩印或者其它方式合理复制学位论文。

本学位论文属于：

（　　）1.经厦门大学保密委员会审查核定的保密学位论文，于　　年　　月　　日解密，解密后适用上述授权。

（　　）2.不保密，适用上述授权。

（请在以上相应括号内打“√”或填上相应内容。保密学位论文应是已经厦门大学保密委员会审定过的学位论文，未经厦门大学保密委员会审定的学位论文均为公开学位论文。此声明栏不填写的，默认为公开学位论文，均适用上述授权。）

声明人（签名）：

年　　月　　日

目录

Contents

摘要

锌（Zn）和双酚 A（BPA）是典型的低毒性环境污染物，已有证据表明它们会对水生生物和人类生殖系统构成潜在威胁。Zn 是机体必需微量元素，但其过量累积又会危害个体健康，其毒理效应机制复杂。BPA 是典型的环境内分泌干扰物，影响生物性腺发育和性别特征，进而影响生物种群性别比例，对环境生态系统造成破坏。而这二者生殖毒性的分子机制目前尚不明确。牡蛎是富 Zn 能力最强的生物之一，具有雌雄同体和雌雄转换现象，性腺发育周期短且高度发达，是研究环境污染物生殖毒性的常用模式生物。本文以福建牡蛎为对象，研究在长期胁迫条件下，$ZnCl_2$ 和 BPA 对牡蛎性腺的慢性毒性效应, 以 ICP-MS 和 GC-MS/MS 方法分别分析牡蛎性腺 Zn 及 BPA 的富集情况，再利用 LC-MS/MS 方法分析胁迫条件下牡蛎性腺全蛋白质组表达谱变化情况以探究 Zn^{2+} 与 BPA 对牡蛎的生殖毒理效应机制。

首先进行了 $ZnCl_2$ 毒理蛋白质组学研究。通过联合运用组织切片、SOD 与 CAT 酶活测定以及 ICP-MS 检测这三种技术，本研究发现伴随着氧化应激对性腺发育所造成的抑制作用的加剧，雌雄牡蛎性腺对 Zn 的富集量不减反增，表明牡蛎性腺富 Zn 水平与其性腺发育程度呈反比关系。这种现象过去也曾被报道过。我们推测牡蛎性腺富 Zn 水平同其生殖周期调控有关。同时，本文蛋白质组的分析结果证实了牡蛎主要通过 Zip 家族、ZnT 家族和金属硫因蛋白/硫因蛋白体系参与调控牡蛎性腺内细胞 Zn 的生理稳态，而且还发现了 cavortin 蛋白可能在维持细胞 Zn 内稳态中也发挥着重要作用。对于其他差异表达蛋白，本文将它们构建成 STITCH（蛋白功能关联）网络，为了便于分析，进一步将全网络细分为几个小的功能网络，涉及能量代谢、氧化应激、基因表达调控等。对 STITCH 网络的分析，本文发现，Zn^{2+} 胁迫后雄性性腺糖代谢紊乱，供能从依赖于糖类转为脂肪动员，而雌性仍然以糖类代谢为主；同时胁迫还造成雄性性腺基因活化，但却使雌性加强了基因沉默; 此外，酶活分析显示雄性性腺抗氧化胁迫酶被强烈抑制，而这些酶的活性在雌性性腺中却逐渐恢复。本文推测，雌雄性腺对 Zn^{2+} 胁迫的应答机制不同，但雌性展示了较雄性更强的适应性。此外，本文还分别从雄性与雌性性腺的差异表达蛋白中筛选出了指示 Zn^{2+} 污染的候选生物标志蛋白，雄性的包括 14-3-3 蛋白、半乳糖基转移酶、丝氨酸/苏氨酸蛋白激酶、半乳糖基转移

酶等，雌性的包括了半椎蛋白、环己二烯脱氢酶、ABC 转运通透酶等。这些差异表达蛋白也有助于进一步认识 Zn 对雄性与雌性牡蛎的生殖毒性机制。

其次，进行了 BPA 毒理蛋白质组学的研究。本文发现 BPA 的毒理学效应与 Zn^{2+}完全不同。组织切片与性腺指数分析共同表明，对雌性而言，1ppm BPA 的慢性胁迫刺激了卵巢的发育，当浓度为 2ppm 时，却显示出抑制效应。而雄性方面，1ppm 与 2ppm 浓度的 BPA 均呈抑制效应。可见 BPA 的生殖毒理效应同它的浓度和施用对象的性别有直接关系。BPA 生殖毒理机制主要同其类雌激素效应相关，它能结合雌激素受体，还可充当转录因子，且当它结合至雌激素受体应答元件时候会对基因表达产生影响，并对激素的生理功能产生干扰。故 BPA 的毒性效应机理因雌雄而异。本文利用蛋白质组学手段进一步发现，对于雌性性腺，BPA 抑制了牡蛎性腺内多种转录延伸因子表达，可能干扰了基因的转录调控；抑制细胞骨架蛋白 tubulin 可能造成滤泡的合成受阻；刺激雌激素受体，激活卵黄蛋白原的表达，展现雌激素作用；通过抑制精细胞自身抗原蛋白的表达，阻挡生殖细胞通过 S 期，可能由此造成卵母细胞大量丢失等。然而，对于雄性牡蛎，机制有所不同，BPA 抑制了骨膜蛋白合成，胶原蛋白合成也因此合成不足，从而可能抑制了雄性精巢输精管的生成，造成雄性性腺松散、萎缩；通过电压依赖阴离子通道蛋白 2 与骨膜蛋白的调控，可能干扰精巢内细胞凋亡的发生；并通过上调精细胞蛋白 Sp17 可能干扰牡蛎性别的分化与精子鞭毛、纤毛结构的形成；BPA 还刺激钙调蛋白的上调，可能影响了精子的发生、精子的活动性，等等。这些雄性与雌性的差异表达蛋白被证实具有成为指示BPA污染的生物标志蛋白的潜力。另外，STITCH 同样成功地应用于本实验的研究，所得的 STITCH 网络也被细分为一些小功能网络，包括能量代谢、氧化应激、基因转录调控以及卵黄蛋白原家族间相互作用等。STITCH 网络分析提示，BPA 胁迫后雄性增强了糖酵解与 TCA 循环的供能，雌性仅上调了 ATP 合成酶的表达； BPA 促进了雌性的转录延伸反应，而对雄性则不明显；受 BPA 类雌激素作用，雌雄性腺的卵黄蛋白原均显著上调表达，而雌性性腺内上调幅度更大。另外，酶活分析显示受胁迫后，雄性与雌性性腺的抗氧化酶活性经抑制后逐步上调，而雌性上调更快；本文推测，BPA 对雌性牡蛎充分发挥了类雌激素效应, 而对雄性则较不明显，BPA 对雄性可能氧化性胁迫效应大于类雌激素作用效应。

关键词：锌和双酚 A；福建牡蛎；毒理蛋白质组学

Abstract

Zn and Bisphenol-A (BPA) are the typical environmental pollutants of lower toxity, and there are lots of evidences showing that they threats aquatic life and human reproductive systems. Zn is an important biological essential element, however, excessive accumulation of Zn in organisms would endanger the health of individual organisms, and the mechanisms of related toxicological effects were complicated. BPA, a typical class of environmental endocrine disruptor, affect biological gonadal development and gender features, thereby affecting the sex ratio of the populated life, then endangering ecosystem health. Currently, the molecular mechanisms of reproduction toxicity resulted from them still keep unknown. The phenomenon of hermaphrodite and sex reversal can be found in oyster, which is one of the organisms that have the best performance in the bioaccumulation of Zn. Meanwhile, their gonads are rich and their gonadal developmental cycle is short. Thus, oysters are often chosen as the model organism to study the reproductive toxic effects of environmental pollutants. In this paper, oyster was also chosen as the model organism to study the toxic effects of chronic exposure of oyster gonad to $ZnCl_2$ and BPA. ICP-MS and GC-MS/MS were used to measure the bioaccumulation of Zn and BPA in oyster gonads. Next, different proteome of oyster gonads under the Zn^{2+} or BPA stress were analyzed by the LC-MS/MS to reveal the mechanism of the related reproductive toxic effects.

Firstly, a toxicoproteomic study was carried out in the Zn^{2+}-exposed experiment. With the combined application of histological section, the measurement of SOD and CAT activities and the ICP-MS technology, the present study identified that the Zn bioaccumulation in oyster gonads still increased while the oxidative stress exerted a more strong inhibiting effects on the gonadal development. All these above suggested that the level of Zn bioaccumulation in oyster gonads was inversely proportional to the degree of gonadal development, which was similar to previous reports. It was

speculated that Zn bioaccumulation in oyster gonads was closely related to the regulation of reproduction cycle. At the same time, according to the current proteomics study, we certified that the Zip family, ZnT family and the metallothionein/thionein systems were in charge of cellular zinc homeostasis in oyster gonads. Additionally, cavortin may also play a key role in the cellular zinc homeostasis. Other differentail proteins indentified in this experiment were used for the establishment of the STITCH（chemical-protein interactions）network. The whole STITCH network could be divided into some small functional networks concerning energy metabolism, oxidative stress and regulation of gene expression, respectively. STITCH networks analysis indicated that Zn^{2+} stress in male oyster gonads resulted in gonads glucose metabolism dysfunction, i.e. the main energy supply of the gonads was switched from saccharometabolism to lipid mobilization, while the main energy supply of female gonads under Zn^{2+} stress was still from saccharometabolism; Zn^{2+} stress induced gene activation in male gonads while it caused gene silencing in female gonads; additionally, enzymatic activity analysis suggested in the male gonads, antioxidant enzyme activity were strongly and persistently inhibited by Zn^{2+} stress， whereas in the female gonads, they recovered gradually after the inhibition. According to these results, we speculated that the responses to Zn^{2+} stress were different between the male and female gonads. By the way, we selected some candidate biomarkers of Zn^{2+} pollution from the differental proteins in the male and female groups, respectively. More specifically, in the male groups, 14-3-3 epsilon, galactosyltransferase and serine/threonine protein kinase can be regarded as the candidate biomarkers, while in the female groups, hemicentin, cyclohexadienyl dehydrogenase and ABC transporter permease also has the potential to be the candidate biomarkers.

Next, the toxicoproteomic study was also performed in the BPA-exposed experiment. The toxicological effects of BPA were different from those of Zn^{2+} exposure. The combination of morphological observation and determination of gonadosomatic index (GSI) showed that the chronic stress of 1ppm BPA accelerated the growth of female gonads while the effects of 2ppm BPA to oyster ovaries were

prohibitive. However, both of the 1ppm and 2ppm BPA showed prohibitive effects to the development of male gonads. All this indicated that the toxicological effects of BPA showed closely related to its exposed concentration and the gender of the objects. In fact, the main mechanism of toxicological effects is closed to BPA's estrogen-like effects. BPA has the ability to combine with the estrogen receptor. Once it finished combining with the estrogen receptor, it will affect the gene expression and disturb the physical function of estrogens. Thus, the toxicological effects of BPA varied by genders. Our current toxicoproteomic study further indicated that, in terms of female gonads, BPA restrained the various translation elongation factors, which may result in the disturbance of gene translation regulation; it also restrained the tubulins, possibly preventing the formation of follicles; moreover it stimulated the estrogen receptor, potentially activating the expression of vitellogenin to show its estrogen-like action; in addition, it can also inhibit the expression of nuclear autoantigenic sperm protein, probably preventing the germ cells entering the S phase, maybe resulting in losing a large number of oocytes. However, for the male oysters, the mechanism was different. Specifically, BPA inhibited the formation of periostin, resulting in the insufficient synthesis of collagen, finally maybe contributing to the inhibition of spermaducts in testis, thus the testis showed to be atrophic; it also regulated the voltage-dependent anion-selective channel protein 2 and the periostin, potentially disturbing the apoptosis in the testis. Furthermore, BPA stress upregulated the sperm surface protein Sp17 possibly disturbing the sexuality differentiation and the formation of spermatic flagellum and cilia. In addition, upregulation of calmodulin induced by BPA possibly affected the formation of sperms and their mobility. Furthermore, these proteins were regarded as the potential candidate biomarkers for BPA pollution. Besides, the STITCH network also was successfully applied in the BPA-exposed experiment and the whole network also could be divided into some small functional networks including energy metabolism， oxidative stress， regulation of gene transcription and the vitellogenins interaction network. STITCH networks analysis suggested that the glycolysis and tricarboxylic acid cycle of the male gonads under BPA-stress were increased which enhanced their energy supply, while the female

gonads only increased ATP synthetase expression level; moreover, enzymatic activity analysis suggested under the BPA stress, the activities of the antioxidant enzymes were inhibited befroe a gradual increase, and the speed of upregulation in female gonads faster than that of the males; BPA stress boosted the transcription elongation of the female gonads but not that of the males; with the stimulation of BPA' estrogen-like effects, vitellogenins were remarkably upregulated in the male and female gonads, with greater level of upregulation observed in the female gonads. It was speculated that the BPA' estrogen-like effects worked obviously in the female oysters, however, these estrogen-like effects have little influences on the males while the oxidative stress effects might be more apparent in the males.

Key words: Znic and bisphenol-A; *Crassostrea angulata*; toxicoproteomics

1 前言

1.1 锌污染研究概况

1.1.1 锌污染概况

锌（Zn）是所有生物的必要微量元素, 它构成了 300 种酶的重要辅助因子，还是大量蛋白质的重要组成成分。此外，理想的核苷酸与蛋白质代谢、细胞生长、细胞分化和细胞功能的发挥都需要足量 Zn 的参与[1]。

与此同时，作为一种常用的重金属，Zn 被广泛应用于人类生产生活中。据统计，在当代人们使用的金属中，按用量计算，锌是仅次于铁、铝、铜之后的第四大用量的重金属。中国作为世界工厂，早已是锌的生产和消费大国。锌广泛使用于冶金、化工等产业，主要被用于电镀钢制品以防止腐蚀,其次是黄铜耗锌、压铸合金等[2]。一方面，Zn 带给人类巨大的利益，另一方面伴随着生产和使用锌的过程，大量的含锌废水不可避免地产生,已经造成了严重的环境污染问题。有数据显示，我国江河湖库底质的污染率惊人地高达 80.1%[3]，国内主要的河流湖泊重金属污染状况令人堪忧，例如，作为长江第二大支流的湘江，重金属污染是其水体污染状况的最显著特征；Zn、Cd 等是其最主要的污染物，直接对该流域 4000 万人口的饮用水安全构成严重威胁。此外，Zn 等重金属污染物也危及湘江的水生生态环境，造成鱼类大幅减少, 废水毒死鱼类的现象频频发生，数以千公倾的农田、土壤及作物不可避免受到污染；其直接原因便是采矿业的发展带动大量有色金属和稀有金属矿藏的开采，并吸引众多冶炼企业齐聚在湘江流域, 它们将工业废水、废渣、废气等的排放入水体，直接造成湘江的严重污染[4]。

Zn^{2+}胁迫对人体和环境直接造成一定的危害，具有持久性、毒性大、污染严重等特点。对人类而言，Zn 有三种主要方式进入人体内，即摄入、皮肤吸收与吸入[1] 。其中，特别值得关注的摄入方式是通过食物链吸收过量的 Zn，Zn 被排放到环境后不能被生物降解, 大多参与了食物链循环, 并最终在生物体内富集, 进而对正常生理代谢活动造成破坏，这种胁迫途径比较隐蔽，不易察觉。

依据 Zn^{2+}对大鼠和小鼠半致死浓度的推算，Zn 对人的半致死浓度约为 27g/