ARTÍCULO ORIGINAL

Network prediction of the potential molecular mechanisms in birds of prey exposed to organochlorine pesticides
Red de predicción de potenciales mecanismos moleculares en aves de presa expuestas a pesticidas organoclorados

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Abstract. In the 1960s, organochlorine compounds were responsible for the decline of birds of prey populations such as *Haliaeetus leucocephalus* and *Falco peregrinus*. Pesticides similar to DDT cause bioaccumulation in birds, affecting their eggshell composition and compromising their development. Using system biology tools, the goal of this study was to better comprehend how organochlorines act on birds. We performed a literature review, using the STITCH 5.0 platform, searching for the terms DDT and TCDD. The sub-networks were amplified in 100 interactions in STRING 10.5 and joined by the Cytoscape 3.4.0 Merge software, using the experimental animal model *Gallus gallus*. Clusterization, gene ontology, and centrality were the parameters evaluated in the resulting network. The resulting network had 1,417 interactions and 137 nodes. The clusterization indicated four *clusters* and the gene ontology pointed to biological processes related to cell signaling and morphological development. The centrality analysis indicated ESR1 and HSP90AB1 as *hub/bottleneck* proteins involved in the estrogen pathway and calcium transport. Therefore, it is possible that HSP90 proteins have increased expression in birds contaminated with organochlorine pesticides, favoring ESRI-organochlorines interaction and disturbing the calcium availability related to the eggshell formation. The presence or absence of heat shock proteins, such as HSP90, influences several aspects of reproduction in many species. Therefore, the relationship between the HSP90 protein expression and thin-shell syndrome was identified for the first time in this *in silico* study.

Keywords: Ligand-protein network; OPIN network; Eggshell; Birds of prey; Pesticides.

Resumen. En los años 60, los organoclorados fueron responsables del declive de aves de rapiña como *Haliaeetus leucocephalus* y *Falco peregrinus*. Pesticidas como el DDT, causan biomagnificación en las aves, afectando las cáscaras de los huevos y dañando su desarrollo. El objetivo de este trabajo fue, a través de herramientas de biología de sistemas, comprender cómo los organoclorados actúan en el organismo de las aves. A través de una revisión bibliográfica se incluyeron dos compuestos, DDT y TCDD. Estos fueron prospectos en la plataforma STITCH 5.0. Las subredes encontradas fueron aumentadas en 100 interacciones en la plataforma STRING 10.5 y unidas por la herramienta Merge del programa Cytoscape 3.4.0, usando el modelo experimental *Gallus gallus*. En la red resultante se analizaron la clusterización, la ontología génica y la centralidad. La red resultante presentó 137 nudos y 1.417 interacciones. El análisis de clusterización indicó 4 *clusters*, siendo que el análisis y ontología génica apuntó procesos biológicos ligados a la señalización y al desarrollo morfológico. El estudio de centralidad apuntó a ESR1 y HSP90AB1 como los *hubs-bottleneck* proteínas que estaban involucradas en la vía de recepción de estrógeno y en el transporte de calcio. De acuerdo con los resultados podemos inferir que las proteínas HSP90 tienen su expresión aumentada, en aves contaminadas con pesticidas organoclorados, favoreciendo la interacción entre ESRI y DDT / TCDD. Con ello, la interacción ESRI y la hormona estrógeno se compromete perjudicando el transporte de calcio y consecuentemente la formación de la cáscara del huevo en aves expuestas. La expresión de proteínas de choque térmico ha sido asociada a varios aspectos de la reproducción en muchas especies, sin embargo, una asociación entre HSP90 y el síndrome de la cáscara fina del huevo fue identificada por primera vez en este experimento *in silico*.

Palabras clave: Red proteína-ligante; Red OPIN; Cáscara de huevo; Aves de rapiña; Pesticidas.

Introduction

In the period between 1950 and 1975, bald eagle populations suffered a large decline, driving the species to a danger of extinction in 43 USA states. This decline was associated with environmental contaminations, especially to the wide use of pesticides such as dichlorodiphenyltrichloroethane

(DDT). After the DDT prohibition, bald eagle populations raised significantly in the United States (Bowerman 1994).

These toxic compounds have slow degradation, persisting for long periods in the environment and can bioaccumulate and biomagnify in successive trophic levels, creating a serious threat to the wildlife and human populations (Elliott *et al.* 2009; Hong *et al.* 2014). The book 'Silent Spring', by Rachel Carson, reported this bioaccumulation process of organochlorines (OCs) and indicated DDT as the cause of the reproductive failure in birds (Carson and Darling 1962).

The DDT use was banned in many countries; however, due to its high persistence, it is still found in the wildlife (García-Fernández *et al.* 2013; Abbasi *et al.* 2016; Espin and col. 2016)but this issue has received less attention in birds. In this article, we reviewed the available literature on levels of legacy persistent organic pollutants (POPs.) Furthermore, in countries such as India, DDT is still the ongoing pesticide in food crops and other economic crops. India has the largest DDT production, representing 84% of the global DDT use in the period of 2001–2014 (Berg and col. 2017). Being a DDT, OCs are known to induce some abnormalities in birds such as mortality or reduced hatchability, failure of chicks to thrive (wasting syndrome), and teratological effects (Fry 1995). Teratological effects produce skeletal abnormalities and impair the differentiation of the reproductive and nervous systems through mechanisms of estrogen-mimicking (Fry 1995).

These compounds interfere in hormonal activities, mimicking estrogen actions and impairing their receptors (Fry 1995). The xenobiotic hormone disruptions are complex, and effects in birds and mammals may be dissimilar due to differences in the estrogens and androgens control during the reproductive differentiation system (Fry 1995). Even though the relationship between OCs exposure and hormonal response disruption is understood, the molecular mechanisms associated with this disruption in birds of prey contaminated with OCs are unknown.

Using *in silico* analysis, the goal of this study is to contribute to a better comprehension of the effects of OCs on birds and how they are affecting their reproduction and causing the population decline.

Material and methods

Interaction Network Construction

Gallus gallus was used as a model organism in a chemical and biological interaction network built to better understand the effects of OCs compounds on the physiological alteration related to the reproduction of birds at a molecular level. To achieve that, we performed a literature review and identified two OCs compounds to which prediction in *G. gallus* is available. Then, we searched for compound-protein interactions in the STITCH v.5.0 database (http://stitch.embl.de) (Szklarczyk *et al.* 2016). The STRING 10.5 search tool (http://string-db.org) was used to find protein-protein interactions (Snel 2000; Kuhn *et al.* 2008; Szklarczyk *et al.* 2017) information on such interactions is widely dispersed across numerous databases and the literature. To facilitate access to this data, STITCH ('search tool for interactions of chemicals'.) The interaction between chemical compounds and proteins in STITCH is based on experimental evidence, databases, and published data (Kuhn *et al.* 2008). The following parameters were used to import sub-networks from STITCH: 50 interactions or less, medium confidence score (0.400), and network depth equal to 2; all prediction methods were activated except for text mining, gene fusion, and co-occurrence. This initial process generated two small compound-protein interaction sub-networks. Then, we searched for protein interactions in STRING 10.5. This web tool predicts protein interactions that can be directly (physically) or indirectly (functionally) associated (Snel 2000). The following parameters were used: no more than 100 interactions, medium confidence score (0.400), and network depth equal to 2; all prediction methods were activated except for text mining, neighborhood, gene fusion, and co-occurrence. The distinct sub-networks generated in these two processes were joined in a single sub-network denominated Organochlorine-Protein Interaction Network (OPIN), using the Cytoscape 3.4.0 Advanced Merge Network software (Shannon *et al.* 2003).

Topological Analysis

OPIN was analyzed by the Cytoscape 3.4.0 plugin MCODE (Molecular Complex Detection) (Bader and Hogue 2003) phage display and mass spectrometry have enabled us to create a detailed map of biomolecular interaction networks. Initial mapping efforts have already produced a wealth of data. As the size of the interaction set increases, databases and computational methods will be required to store, visualize and analyze the information in order to effectively aid in knowledge discovery.|This paper describes a novel graph theoretic clustering algorithm, \"Molecular Complex Detection\" (MCODE aimed at identifying modules/clusters (densely connected network regions)

that suggest functional protein complexes. The following parameters were applied to MCODE to generate clusters visualization: loops included; degree cutoff 2; one connection node exclusion (haircut option activated); cluster expansion by one neighbor shell allowed (fluff option activated); node density cutoff 0.1; node score cutoff 0.2; kcore 2; and maximum network depth 100. An MCODE score was calculated for each OPIN protein/compound.

Gene Ontology Analysis

The main processes associated with the clusters generated by the MCODE were analyzed by the Cytoscape 3.4.0 plugin BiNGO (Biological Network Gene Ontology) (Maere *et al.* 2005). The equation degree of a given cluster and category was quantitatively computed (*p*-value) by hypergeometric distribution. Multiple correction tests were applied using the false discovery rate algorithm (Benjamini y Hochberg 1995) researchers, and students discover, use, and build upon a wide range of content in a trusted digital archive. We use information technology and tools to increase productivity and facilitate new forms of scholarship. For more information about JSTOR, please contact support@jstor.org. SUMMARY The common approach to the multiplicity problem calls for controlling the familywise error rate (FWER performed by BiNGO with a 5% significance level.

Centrality Analysis

Centrality analysis was performed using the Centiscape 2.1 plugin (Scardoni *et al.* 2009) and Cytoscape 3.4.0 to identify the nodes (proteins) with a central position within the network. Node degree and betweenness were the analyzed centralities; nodes with a relatively high degree are named 'hubs' and nodes with a relatively high betweenness are named 'bottlenecks'. Hubs are highly connected nodes, while bottlenecks are more probable to connect different clusters (Barabasi and Oltvai 2004; Yu *et al.* 2007). Therefore, a hub-bottleneck node (HB-N) can be considered a key regulator of biological processes, extremely important for successful information transferring within the network. Hence, disturbances in an HB-N can cause communication failures within the network.

Results and discussion

In recent years, the increasing number of "omic" databases has enabled the use of computational tools to understanding the interplay between the regulatory processes acting on a cell (Feltes *et al.* 2011). Based on previous studies that associated OCs exposure to reproductive failure in birds (Carson and Darling 1962), we built an organochlorine-protein network interaction to investigate and to propose the molecular mechanism associated to the effect of organochlorines on birds.

Our OPIN network had 1,417 interactions and 137 nodes (*Figure 1*), of which 135 are *G. gallus* proteins and two are OCs compounds (DDT and 2,3,7,8-tetrachlorodibenzo-p-dioxin — TCDD) prospected from the literature review. Due to the scarce STITCH database about OCs compounds, only two compounds were prospected. In relation to network analysis had: clustering coefficient equal to 0.541, diameter to equal 4, radius equal to 3, centralization equal to 0.45, the shortest paths equal to 18632, characteristic path length equal to 2,172, avg. number of neighbors equal to 20,686, density equal to 0.152, heterogeneity equal to 0.669 and closeness equal to 0.0034.

The MCODE analysis resulted in four clusters, a set of nodes connected to each other, very common in biological systems since cellular processes are ruled by biomolecules that form interaction modules (Pavlopoulos *et al.* 2011. The BiNGO plugin indicated that the main metabolic processes associated to the cluster 1 (cluster with the highest score) are involved in the reproductive success and in the development of organs and tissues (*Table 1*). Hub-bottleneck proteins are nodes with the highest relevance inside a network and have a large number of connections capable of linking distinct clusters. The four main HB-N (UBB, ESR1, and HSP90AB1 proteins and organochlorine TCDD) found by the centrality analysis are shown in *Figure 2*.

TCDD was found with one of the main hub-bottleneck proteins because it is able to connect a large number of clusters within a network, evidencing the great impact of this compound in a biological system. UBB is known as polyubiquitin b and plays an important role in proteins degradation, but also acts as a regulator of several biological mechanisms such as gene expression and stress responses. UBB is a highly conserved protein that plays critical roles in the functioning of eukaryotic cells (Hochstrasser 1996) and is frequently present as a HB-N in the system biology.

The other two main HB-N found in the analysis were the proteins ESR1 and HSP90AB1. ESR1 (estrogen receptor 1) is responsible for the estrogen reception in the cell. Once bound, the hormone and its receptor activate a series of signaling events involved in the genetic regulation that affect tissues and cellular proliferation (www.uniprot.org/uniprot/P06212) (Bateman *et al.* 2017)

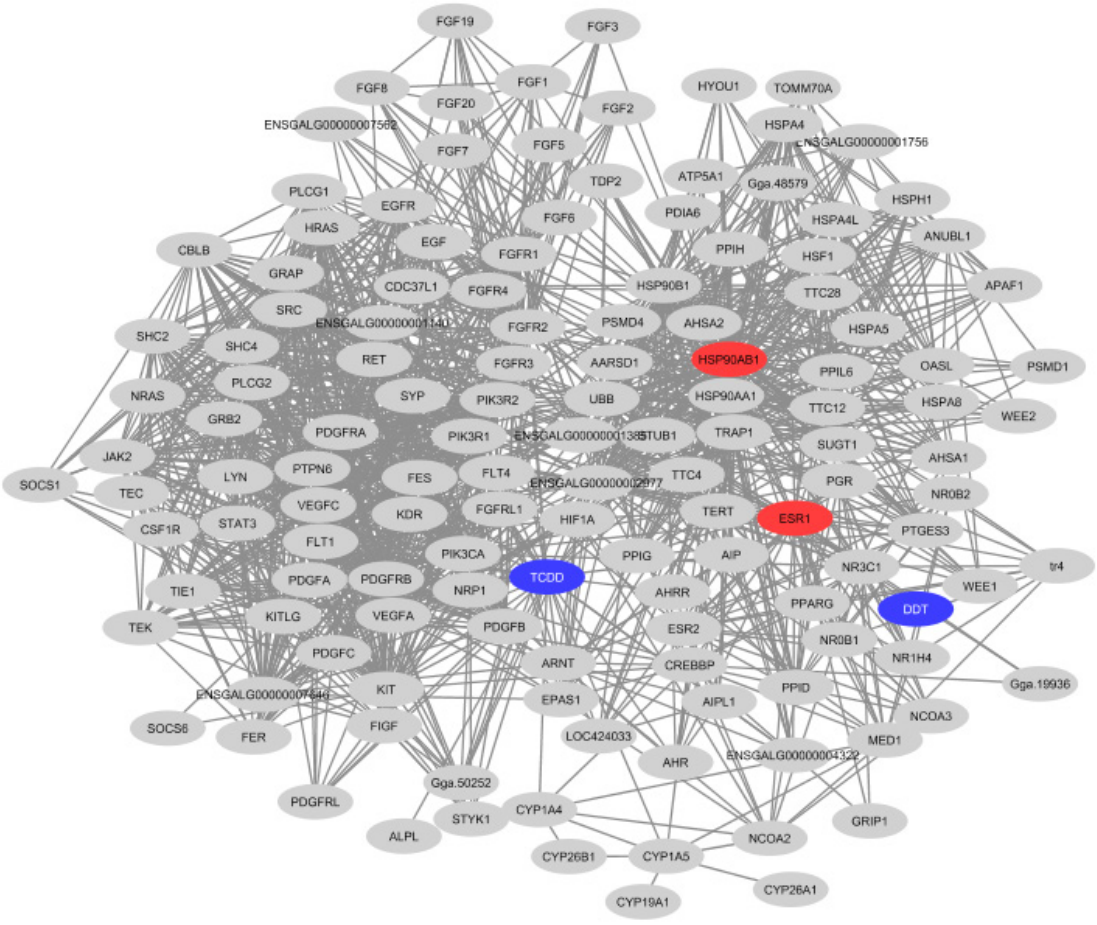

Figure 1. Organochlorine-protein interaction network (OPIN) of the model *Gallus gallus* with 137 nodes and 1,417 edges. The blue nodes represent the chemical compounds DDT and TCDD, grey nodes represent the proteins, and the red nodes represent the two main hub-bottleneck proteins. The edges between nodes represent the interaction display of this biological system.

(ESR1 UniProt code: P06212). HSP90AB1 is a chaperone protein that promotes the regulation of specific targets involved in the cell cycle and signal transduction, leading to a conformational change in target-proteins, causing their activation (HSP90AB1 UniProt code: Q04619). According to Fliss *et al.* (2000), HSP90 acts on maintaining the estrogen receptor in a high-affinity hormone-binding conformation, ensuring that the ESR1-hormone system performs the actions resulted from the hormonal activity. Animals exposed to negative environmental conditions, such as pesticide exposure, have been described to exhibit increased levels of HSP40, HSP70, and HSP90 (Brom *et al.* 2015).

DDT and other OCs have mimicking action; they are capable of binding to the same receptors as estrogen, including ESR1. Once DDT compounds bind to estrogen receptors they can activate them, generating a signaling event cascade and the synthesis of certain cellular products (Jaga 2000).

The best-documented case of the effects of environmental pollutants on birds is the thin-shell syndrome caused by dichlorodiphenyl (DDE) — a chemical derivative of DDT — particularly in species that feed on fishes, leading to a reproductive failure (Gress *et al.* 1973). The species more susceptible to the thin-shell syndrome appear to have a reduced ability to metabolize organochlorines (Schwarzbach *et al.* 1991) p' dicofol, p,p' dichlorobenzophenone (p,p' DCBP. Moreover, estrogen and thyroid hormones that control calcium availability are involved in the eggshell formation (Hoffmann and Völker 1969).

Thus, birds contaminated with OCs pesticides probable have an increased expression of HSP90 that inhibits the hormone-binding conformation and consequently favors ESRI-OCs interaction.

Table 1. Gene Ontology (GO) classes derived from the cluster 1 network. GO identification number (ID), the significance value of each process, the bioprocess of each cluster, and the proteins involved are displayed. The main hub-bottleneck proteins are displayed in bold letters.

GO - ID	p-value	Biologicalprocess	Proteins
7167	2.86 x 10^{-15}	Signaling of the receptor protein bound to an enzyme	RET|PDGFRB|NRP1|PDGFRA|CSF1R|FIGF|EGF|TIE1|STAT3| PDGFB|PDGFA|FGF1|EGFR|VEGFA|KIT|PDGFC|STUB1|GRB2| TEK|JAK2|FGFR3
23033	1.97 x 10^{-07}	Signalingpathway	RET|SHC4|NRP1|CSF1R|FIGF|SHC2|PDGFB|PDGFA|CBLB|FGF1| EGFR|NRAS|PDGFC|PLCG2|JAK2|HRAS|PDGFRB|LYN|PDGFRA| EGF|TIE1|STAT3|ESR2|VEGFA|TEC|PIK3CA|KIT|PGR|PTPN6| STUB1|GRB2|TEK|FGFR3
48856	3.08 x 10^{-07}	Anatomicstructuredevelopment	RET|NRP1|FLT1|**HSP90AB1**|SRC|EPAS1|PDGFB|PDGFA|FGF1| FGFRL1|NRAS|PDGFC|KDR|JAK2|HRAS|LYN|PDGFRA|EGF| STAT3|ARNT|**ESR1**|ESR2|VEGFA|KITLG|KIT|PGR|GRB2|FGFR3
48731	1.95 x 10^{-06}	System development	RET|NRP1|FLT1|**HSP90AB1**|SRC|EPAS1|PDGFB|PDGFA|FGF1| FGFRL1|PDGFC|KDR|JAK2|LYN|PDGFRA|EGF|STAT3|ARNT| **ESR1**|ESR2|VEGFA|KITLG|KIT|PGR|GRB2|FGFR3
9653	2.11 x 10^{-05}	Morphogenesis of anatomicalstructure	RET|NRP1|PDGFRA|FLT1|SRC|EPAS1|EGF|STAT3|PDGFB| PDGFA|FGF1|**ESR1**|FGFRL1|ESR2|VEGFA|PDGFC|KDR|PGR|GRB2|FGFR3

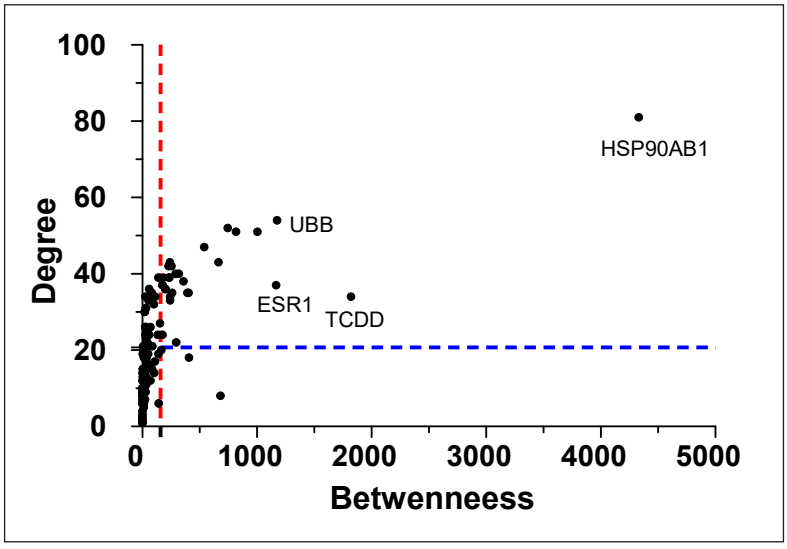

Figure 2. Hubs-bottlenecks (HB) graph of the most important network nodes. The horizontal axis shows the betweenness values and the vertical axis shows the degree values. The mean of each centrality is represented by the dashed lines. The dots beyond the dashed lines are the HB nodes, highlighting the proteins ESR1, HSP90AB1, UBB, and the TCDD organochlorine.

The result of the disrupted ESR1-estrogen interaction is the calcium unavailability for eggshell formation, which leads to the thin-shell syndrome (*Figure 3*). As previously described, the syndrome is related to the calcium inhibition in the eggshell gland by DDE, an organochlorine very similar to DDT (Kolaja 1979). Derfoul *et al.* (2003), using human trophoblast cells, demonstrated that calcium handling is probably under estrogenic regulation and OCs functionally perturbs the trophoblastic calcium transport. The authors affirmed that this perturbation may result (i) in the inhibition of the activities of cellular components involved in the calcium transport and (ii) in the interference with

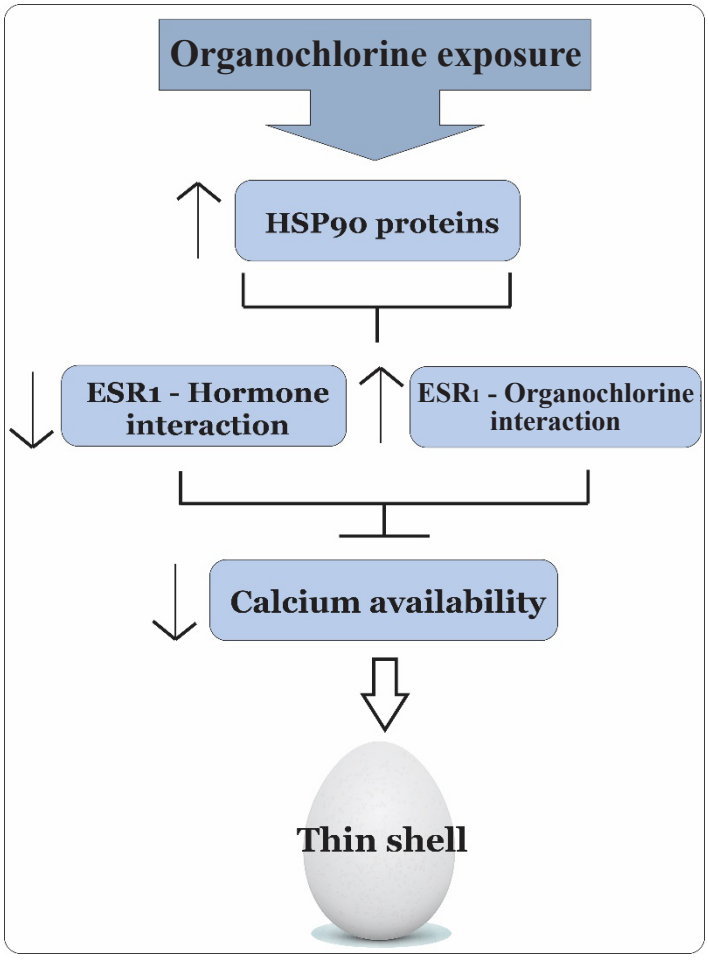

Figure 3. A molecular model illustrating the effect of organochlorines on exposed birds. The organochlorine exposure probably leads to an increase in HSP90 proteins expression, inhibiting the hormone-binding conformation and consequently favoring the ESRI – organochlorine interaction. Thus, the disruption of the ESR1- estrogen interaction creates a calcium unavailability for eggshell formation, causing the thin-shell syndrome.

the estrogen-regulated calcium transport pathway. Furthermore, some studies provided novel insights into the roles of HSP90 and calcium transport. Li *et al.* (2014) observed that HSP90 affects the intracellular calcium homeostasis in human sperm. Organochlorine compounds were banned in the USA in the early 1970s. Even so, DDT and other organochlorine compounds are still found in eggs and carcasses of birds and in birds inhabiting urban and suburban areas (Espín 2010; Martínez 2015; Mora and col. 2016; Yohannes 2016; Schmitt and col. 2018). Organochlorine contamination has been recorded worldwide, in Europe (Espín 2010), Africa (Yohannes 2016), and South America (Martínez 2015). This may be occurring due to the bioaccumulative feature of these compounds; they remain in the food chain of ecosystems, including human's. Since there are countries that continue to use these compounds on a large scale (Berg *et al.* 2017), they are still a threat to the wildlife.

Conclusion

Organochlorines have a high toxic potential to all organisms due to their bioaccumulation capacity and for being estrogenic. In this study, the results of clusterization associated to gene ontology pointed to biological processes related to cell signaling and morphological development. The centrality analysis indicated ESR1 and HSP90AB1 as hub/bottleneck proteins involved in the estrogen pathway and calcium transport. Thus, we proposed that the estrogen pathway related to chaperone proteins (HSP90) was the prospected molecular process by which these compounds act and their dysfunctioning affects the calcium availability for eggshell formation. The presence or absence of heat-shock protein, such as HSP90, influences several aspects of reproduction in many species. Therefore, the relationship between the expression of HSP90 protein and thin-shell syndrome was identified for the first time in this study.

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