

Title:

Data transfer from animal models to human studies: what is the challenge? Case of persistent organohalogenes.

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Abstract:

Decision making in the sphere of regulation of number of environmental contaminants was done with the delay averaging 50 years. We hypothesised that one of the causes of that delay is the low coherence in studying of toxic effects in animal models and human studies. To test this hypothesis we analysed abstracts and free full texts of all PubMed published studies of toxic effects of polychlorinated biphenyls (PCB) and polybrominated diphenyl ethers (PBDE) in animal models (807 in total). The analysis demonstrates that human studies cannot gain in big experience from the studies of animal models because of the following reasons: 1) significant delay in start of experimental research; 2) use of exposure levels several orders higher than exposure of general population; 3) shortage in using of animal species with toxicokinetics, physiology of development and pregnancy similar to human; 4) limited set of endocrine endpoints, which is predetermined rather by tradition than by other reasons; 5) testing of most sensitive stages of development in the last place (according to the sequence adult animals – neonates – foetuses); 6) lack of experimental studies aimed at search of outcomes suitable for epidemiological studies. Mostly the same disadvantages are characteristic for PCB and PBDE studies showing that no lessons were drawn from history. Broadening of the dialog between different branches of toxicology is needed to accelerate data accumulation necessary for decision making.

Introduction

The history of research of number of environmental contaminants (lead, PCB, methylmercury, Bysphenol A) shows serious delay in accumulation of data necessary for decision making in the sphere of toxic substances regulation [1-3]. These delays averaging 50 years brought huge damage in human health, economy of society and natural ecosystems [4]. In regard to rapid accumulation of industrial contaminants with unknown influence to human health in biota acceleration of decision making process is becoming an ultimate goal of toxicology.

We hypothesise here that at least partly the delay in decision making occurs due to the low coherence in studying of toxic effects in animal models and human studies. Animal models has important role in studying of toxic effects of hazardous substances and determination of dose-response relationships and most susceptible stages of development. Outcomes of experiments with animal models form input of epidemiological studies of human population and decision making process. Therefore, organisation of experiments with animal models can whether accelerate process of decision making or delay it for indefinite period.

To find out if organisation of experiments with animal models influence the speed of accumulation of data we analyse here history of animal studies of one group of old and relatively well studied organohalogen contaminants – polychlorinated biphenyls (PCB) and much shorter history of studies of relatively new group of persistent organohalogen contaminants – polybrominated diphenyl ethers (PBDE). In this review we want also to

answer to the question, if it is possible to draw lessons from the history of already well studied substances, and if we really do draw lessons?

We have analysed abstracts and free full texts of articles that are selected in PubMed after the word PCB or PBDE being typed as search criteria on May 15, 2008. We have analysed total number of articles dealing with experiments with animal models with exception of pure toxicokinetic experiments. No any other restrictions were applied. From total of 6076 PCB articles 748 were analysed and 59 from total of 649 PBDE articles.

In order to analyse if existing animal experiments are coherent to human studies we formulated first the requirements to “harmonized animal experiment” fitting best the goal of transferability of obtained results to human studies. These requirements were derived on the base of reflections concerning design of our ongoing epidemiologic investigation on environmental contaminants. The subsequent analysis of PCB and PBDE research was carried out to evaluate the extent to which the real studies of animal models correspond to the proposed criteria of “harmonized animal experiment”.

To what criteria the “harmonized animal experiment” has to correspond in order to be coherent to human studies?

If consider animal studies as the stage of toxicological activity aimed at making in time reasonable decisions in the sphere of toxic substances policy, the harmonized experiments with animal models have to correspond to the following requirements:

1. The experiments have to be done in time, i.e. long before the substance become a public health problem and enough before to leave enough time for decision making.

This requirement is the most important for the realisation of precautionary approach.

2. Use of doses corresponding to human exposure. Any toxic effects as well as any biomarker of effect obtained at doses of exposure several orders higher than the level of exposure of general population can not be transferred directly to studies of general population as most of toxic substances reveal different effects at different doses.

Moreover, toxic effects obtained in dose-response studies at high doses of exposure can not be extrapolated to the existing low doses of exposure of general population as number of environmental toxicants reveals non-linear dose-response relationships.

Non-monotonic dose response curves were already reported for PCB [5-7] and for PBDE [8, 9]. Biphasic and hormetic dose response curves are widely discussed in toxicological literature [10, 11] suggesting in majority of cases involvement of heterogeneous mechanisms of response at different threshold concentration. Presence of experiments testing high doses only causes false confidence in safety of general population therefore.

In short, using of doses of exposure exceeding several fold exposure of general population does not contribute seriously to understanding of risks for general population.

3. Use of models with similar toxicokinetics and physiology. It is obvious that differences in toxicokinetics predetermines different toxic effects in different species [12]. It is becoming clear in the years that pre- and neonatal stages of development are the most susceptible for number of toxic substances [3]. Therefore, it is important to

study not only animal models with similar adult physiology and toxicokinetics but also imitating human pregnancy, pre- and neonatal development.

4. Determination of most sensitive endpoints. Adequate assessment of risk for general population could be based only on the analysis of effect of toxic substances on the most sensitive target organs and functions. Therefore, comprehensive screening of most sensitive endpoints is necessarily requirement to animal studies.

5. Determination of most sensitive stage of development. As if number of toxicants are known as being developmental toxicants at low doses and do not reveal toxic hazardous properties for adults at the same concentrations, lack of knowledge of the most sensitive stages of development can cause underestimation of risk in regards to most susceptible populations.

6. Determination of outcomes to explore in epidemiological research. Biomarker epidemiology is undergoing rapid development and expansion and is becoming one of the most promising areas of environmental research [13]. Animal studies possess broader possibilities in the search of endpoints of toxicity than epidemiological research of human population allowing sacrifice of animals at any stage of development, tissue harvesting and different types of intervention. Therefore, harmonized animal study has to produce a broad range of outcomes as well as set of peripheral biomarkers of exposure and effect suitable for human studies.

7. Estimation of internal dose. In order to transfer correctly information from animal to human studies the choice of the paradigm of exposure has to be verified by measurement of internal dose. It is especially important as the conditions of exposure of

general population (lifetime, low dose, mixed) can be hardly completely simulated in experimental conditions.

We limit ourselves by 7 requirements for the “harmonized animal experiment” although this list can be vastly extended if considering requirements in more details or involving more experts in its preparation. We suppose however that our analysis of correspondence of real animal experiments with ideal requirements will reveal the level of coherence of animal and human studies.

We decided also to include route of exposure to our analysis of PCB and PBDE studies as route of exposure is intensively discussed in peer review process according to our personal experience, experts not revealing constancy in opinions.

It is commonly accepted that study of mechanisms of toxicity increases greatly value of studies of animal models. Being very interesting itself knowledge of mechanism has very slight influence on decision making. For instance after approximately 30 years of intensive research we still do not have clear understanding of specific mechanisms of toxicity of PCB. This shortage in knowledge could not prevent banning of PCB in 1970s, when its toxic effects become clear. Therefore we do not include analysis of mechanisms in the list of requirements to “the harmonized animal experiment”.

Short history of PCB and PBDE

Both PCB and PBDE are ubiquitous persistent organohalogenes, bioaccumulating in animals which were (PCB) or currently (PBDE) produced in high volumes.

PCB was first manufactured commercially in 1927. It was used as coolants and insulating fluids for transformers and capacitors, stabilizing additives in flexible PVC

coatings of electrical wiring and electronic components, pesticide extenders, cutting oils, flame retardants, hydraulic fluids, sealants (used in caulking, etc), adhesives, wood floor finishes, paints, de-dusting agents, and in carbonless copy paper. Manufacture peaked in the 1960s. Estimates have put the total global production of PCBs on the order of 1.5 million tons [14]. In 1966, they were determined by Swedish chemist Dr. Soren Jensen to be a ubiquitous environmental contaminant [15]. According to estimates by early 1970s global PCB atmospheric emission reached its maximum of almost 400 tons per year [16] and only GE had dumped 0,5 to 1.5 million pounds of PCBs in the Hudson River by 1976 [17].

The toxicity associated with PCBs was recognized very early due to a variety of industrial incidents [18]. The most commonly observed health effects in people exposed to PCBs are skin conditions such as chloracne and rashes. Studies in workers exposed to PCBs have shown changes in blood and urine that may indicate liver damage. In 1968 in Japan, PCB contamination in rice bran oil caused a mass poisoning known as Yushō Disease in over 14000 people [19]. Common symptoms included dermal and ocular lesions, irregular menstrual cycles and a lowered immune response, fatigue, headache, cough, and unusual skin sores. Poor cognitive development in children was reported at much lower doses [20, 21].

PCB production was banned in most of the countries in 1970s. Thus the decision making were done with 50 years delay.

PBDE is a group of flame retardant chemicals which are added to synthetic polymers. It could be found in various building materials, electronics, furnishings, motor vehicles, plastics, polyurethane foams, and textiles. PBDE are being produced as flame

retardants since the early 1970s. The total world demand for the highest production volume PBDE in 2001 was estimated by the bromine industry at 67,440 metric tons [22].

PBDEs were first detected in the environment in 1979 [23] and in biota in the 1980s [24]. Studies examining trends of PBDE levels in North American wildlife have found sharp increases over the period of 20 years, with PBDE levels doubling every 3-5 years [25-28]. In human blood, milk, and tissues, total PBDE levels have increased exponentially during the last 30 years doubling each 5 years [29].

Toxic health effects of PBDEs are not yet well studied, but PBDEs have been associated with neurodevelopmental toxicity and thyroid hormone imbalance in rodents [30-35].

The European Union banned the use of penta-and octaBDE in 2004 and deca-BDE in 2008. In 2004 penta-and octaBDE were withdrawn from the North American market [36]. Deca-BDE still remains in use in North America, moreover little is known concerning PBDE production in Asian countries. Waste and recycling sites, indoor use of PBDE-containing products, and global circulation of PBDE toward the Northern hemisphere from countries without policy, all contribute to long-time persistence of PBDE in densely populated Europe and North America.

Do we do experiments in time?

The first two experimental studies of PCB that could be found in PubMed were published in 1971 year. Since that moment number of articles published per year increases gradually (Fig 1A). It is important to mention that the number of PCB-articles continue to increase after PCB being banned (USA – 1976, 1970s – most of the

countries-producers). Two well pronounced waves of increased interest can be noted with 10-years drop in between of 1984 and 1994. Presumably the first wave of interest was triggered by current exposures and observed toxic effects, while the second – by emerging results of long-term epidemiological studies of general population.

The first two experimental studies of PBDE that could be found in PubMed were published in 1980 year – several years later of PCB banning. In spite of these articles showing toxic effect of PBDE and in spite of known structural similarity of PBDE and PCB, the 20-years long period of almost full lack of interest to PBDE followed after 1980 with only two articles published – one in 1994 and another in 1996 (Fig 1B). The real raise of interest to PBDE started since 2001 and continues until now. Meanwhile concentration of PBDE in Swedish Brest Milk increased approximately one order of magnitude by 1980 [29] and relatively high concentrations of PBDE were found in almost every human and biota samples by early 1990th. Hence, intensive research of PBDE started only when it became ubiquitous and when its concentration became really high in biota.

Do we use doses corresponding to human exposure?

Within more than 30 years period the average daily PCB doses used in experiments with animal models decreased gradually, starting from hundreds of mg per kilo of bw in early 70th and coming now to hundreds of micrograms (Fig 2A). At the same time human dietary daily doses at the period of highest exposure of general population were estimated by US Agency for Toxic Substances and Disease Registry (ATSDR) as tens of nanograms. And now daily exposure of general population is

estimated as picograms per kilo of bw. It means that even now, after 30 years of decrease of PCB doses, experiments with animals still use doses several orders higher than these of human exposure. Appearance of the graph does not change if use average total dose, instead of average daily dose (data not shown).

One can suppose that this lesson of PCB study could turn PBDE researchers to start experiments from doses close to human daily exposure. On the contrary we can see (Fig 2B) again start from high doses (tens of mg) and smooth decrease of dose until reaching threshold of hundreds of micrograms in 2005. Afterwards the doses began to raise again, even that threshold being far above the exposure of general population: daily PBDE dietary intake of U.S. population was estimated by Schecter et al [37] as 0.2 µg/kg bw for nursing infants and 0.001 µg/kg bw for adults.

We hypothesized that increase of doses after 2005 occurs due to achieved no observed adverse effect level (NOAEL) at doses used in 2005. To test this hypothesis we made several developmental experiments with rat and sheep animal models exposed subchronically to low doses of PBDE.

Pregnant rats were exposed to vehicle or low dose of most prevalent in human samples PBDE congener BDE-47 (0.002, 0.02 and 0.2 mg/kg body weight) each 5 days from gestation day 15 to postnatal day 20 by intravenous injections. Several endocrine and behavioural endpoints were addressed in pups [38]. Pregnant sheep were exposed to vehicle or BDE-47 (0.2, 2 and 20 µg/kg of body weight) 5th to 15th weeks of gestation by intravenous injections weekly. Thyroid hormones were analysed in lambs upon delivery. BDE-47 content in adipose tissue of both models (mothers and offspring) was analysed and was similar to that known for human populations. The observed effects are

summarised in Table 1, indicating that even doses several fold lower than those achieved in 2005 produce number of adverse effects. We conclude therefore, that it was not the case of achieved NOAEL which turned investigators to increase levels of exposure in animal models, but more likely fear of obtaining of unpublishable results.

Do we use appropriate species?

The diversity of big taxonomic groups used as animal models and mammalian models in more details are shown in figure 3 for both PCB and PBDE studies. First, one can see some increase in diversity of big taxonomic groups used in experiments with animal models in the last years, although, there is opposite tendency within mammalian models.

Rodents are becoming almost only mammalian order used in toxicological experiments. In spite of great number of well known advantages of rodents as model organisms, this group of organisms differs from humans in number of parameters especially important for the study of developmental toxicity [39], for instance physiology of pregnancy, pre- and neonatal development and toxicokinetics of organohalogenes [40].

Obviously primates are the best model to simulate human physiology, development and toxicokinetics, one can see however that we have almost lost primates as model group. This is reasonable from ethical and financial points of view. But the problem is that no any other group having similar to human parameters appears in toxicological studies. Presumably some mammals of Artiodactyla order can be good models imitating human pregnancy pre- and neonatal development and toxicokinetics

[41]. Unfortunately there are few experiments with hoofed mammals within PCB studies and total lack within PBDE studies.

Do we succeed in search of most sensitive endpoints?

Each new environmental contaminant poses new quest concerning most sensitive endpoints of its toxicity. In spite of knowledge accumulated by toxicology, predicting of toxic properties of new contaminants on the base of their chemical structure remains difficult and not yet resolved task for today. Therefore, intensive screening of most sensitive endpoints is necessary for each potentially hazardous substance. A powerful but not yet well understood tools of “omics” appeared in last decades which could probably intensify search of sensitive endpoints of toxicity in future. Until now two articles were published (both in 2007) studying PCB toxicity by the use of microarray technology and one article studying PBDE toxicity using proteomics was published in 2006. In all other articles different endpoints were tested by traditional methods.

We used slightly modified classification of health effects used in ATSDR Toxicological Profiles for classification of studied endpoints of PCB and PBDE toxicity. All effects were classified among following groups: mortality, respiratory, cardiovascular, gastrointestinal, haematological, musculoskeletal, hepatic, renal, endocrine, dermal, ocular, metabolic, other systemic, immunological and lymphoreticular, neurological, reproductive, developmental effects, cancer and genotoxicity. Following modifications were done: 1 – we consider endpoints dealing with endocrine activity of reproductive organs as endocrine effects rather than reproductive; 2 – we assign all endpoints addressing different organs at early stages of development to

the effect corresponding to the organ rather than developmental and consider as developmental only growth changes, developmental malformations, developmental landmarks and endpoints addressing foetal development.

In general profile of endpoints addressed within PCB and PBDE studies is similar (fig 4). The range of endpoints is broad for PCB studies while experiments addressing cancer, dermal gastrointestinal, haematological musculoskeletal, ocular, renal and respiratory effects are missing in PBDE studies. That fact could be explained just by shorter history of PBDE studies and smaller total number of experiments.

Surprisingly, in spite of big number of experiments testing carcinogenicity of PCB, there is still missing information on carcinogenicity of PBDE in animal models. Although there are number of studies indicating that some congeners exhibit aryl hydrocarbon receptor (Ah-R)-mediated effects and are potent inducers of ethoxresorufin-o-deethylase (EROD), there is still no any direct study of carcinogenic properties of PBDE.

As organohalogenes are well known now to reveal endocrine disruptive properties, we consider endocrine endpoints in more detail (fig 5). The number of studies of endocrine endpoints is big, but the diversity of endpoints itself is limited and are under some strange fashion, same for PCB and PCBE studies. For instance there are a huge number of experiments addressing hypothalamo-pituitary-thyroid axis (202 for PCB and 98 for PBDE studies), but just few studies addressing hypothalamo-pituitary-adrenal axis (22 for PCB and 8 for PBDE studies) while adrenal gland is secreting set of hormones responsible for regulation of almost every process in organism. Moreover, understanding

that adrenal gland is the target for environmental PCB [42], did not stimulate the intensive research of PBDE adrenal effects.

One can hypothesize that adrenal gland is not sensitive to organohalogens. To testify this hypothesis we have studied adrenal endpoints of low dose PBDE toxicity in frames of rat developmental model described earlier. The results of our experiments show (see Table 1) that adrenal gland is probably among most sensitive targets of PBDE toxicity. Hence, lack of attention of scientists to adrenal gland (in contrast to thyroid gland) could be rather explained by tradition then by some other reasons.

Numerous studies of both PCB and PBDE found out correlation between level of exposure and different biomarkers of effect which theoretically could be used in human studies. More detailed analysis of these outcomes indicates following limitations: 1) the range of peripheral biomarkers of toxicity is limited; 2) overwhelming majority of experiments use exposure levels several fold higher than exposure in human population. Emerged outcomes are therefore rather effects of acute toxicity than effects of chronic low dose exposure characteristic of general population. 3) Most of outcomes are produced after sacrifice of animals and are inapplicable to human studies. Measurements of numerous hepatic enzymes activity are of that type for example.

Finally, after analysis of 807 PCB and PBDE experimental works we did not find any study specially designed and aimed at search of outcomes suitable for epidemiological studies. This situation further illustrates lack integration of different branches of toxicology.

Are we effective in search of most sensitive stages of development?

It was shown on number of environmental contaminants including PCB and PBDE that early stages of development are much more sensitive than adults [43, 44]. That knowledge presupposes top-priority of experiments aimed at search of most sensitive stages of development in order to identify potentially most vulnerable part of human population.

The results indicating ratio of developmental *versus* non-developmental experiments per year are summarized in figure 6. One can see that % of experiments using developmental models instead of testing adult animals increase gradually in both PCB and PBDE studies. Moreover, the number of pre- and perinatal exposures *versus* neonatal exposures increases within PCB developmental experiments. The number of PBDE articles is too small to find out any similar well pronounced trend.

Hence, obtained results show that we first test less susceptible adults then more susceptible neonates and finally most susceptible fetuses. We suppose that opposite sequence would be more logical.

What is the internal dose in exposed animals?

Choice of dosing paradigm is a challenge. Estimation of internal dose is therefore means of “quality control” and gives valuable information for transfer of data obtained in experiments with animals to human studies [45].

The data on estimation of internal dose in PBDE and PCB studies are summarised in figure 7. It seems that it is the only case when lesson was derived from history of investigation of PCB and transferred to PBDE studies. In PBDE studies popularity of

internal dose measurement is increasing rapidly while it is decreasing slowly in PCB studies.

What route of exposure is better?

There are two major routes used in experiments with animals: oral and via injections (subcutaneous, intravenous and intraperitoneal). Both routes have some advantages and disadvantages. Main advantage of oral route of exposure is better imitation of conditions of human exposure (at least for organohalogenes), while injections allow better control internal dose, especially for low doses. According to the data summarised in figure 8 there is a clear tendency to transfer from oral exposures to exposure via injections in PCB studies. It is strange that only 4 articles were published using injections in PBDE studies. It looks as if there are two different fashions in PCB and PBDE studies.

Conclusion

Critical analysis of existing experiments testing toxicity of PCB and PBDE on animal models shows that at least in part organisation of toxicological experiments are responsible for the delay in decision making concerning toxic substances as producing knowledge hardly transferable to human studies. Moreover, analysis of history of animal studies shows that mode of actions did not change seriously in the sphere of experimental toxicology after the sad history of PCB arrived. Unfortunately the same disadvantages are characteristic for the studies of PBDE indicating that lessons were not drawn from the story of PCB.

One important inconsistency between animal and human research was shown recently in a critical review of PCB experimental studies [45]: there is the relative lack of animal data on several of those persistent congeners, which are currently used as a measure of human exposure while experimental studies in animals are frequently conducted with commercial PCB mixtures, a test design that does not reflect the exposure situation in humans.

Our survey highlights what important lessons we can draw for the human studies to gain in big experience from animal models. They are following:

- Intensive study of animal model has to start immediately upon arrival or better before arrival at the market of new substance but not when contamination of environment and human population by chemical with unknown hazardous properties become real public problem. As research activity is dependent on the founding this lesson is relevant not only for toxicologist but for organizations providing financial support.
- Toxic effects in experimental studies are obtained at levels of exposure several orders higher than exposure of general population preventing direct transfer of obtained results to human studies. Hence, there is need in studies of toxic substances at low doses relevant for estimated human exposures accompanied by estimation of internal dose.
- There is shortage in using of animal species with toxicokinetics, physiology of development and pregnancy similar to human. Rodents are becoming almost only model used in toxicological experiments. Search of toxic effects especially in developmental toxicology should be extended to other mammalian species.

- For endocrine disruptors all endocrine glands have to be addressed. This is particularly relevant for increasing endocrine problems such as childhood obesity and diabetes mellitus for which the role of environmental contaminants could be suspected.
- According to existing practice the search of the most sensitive stages of development occur in the following sequence adult animals – neonates – foetuses while opposite sequence is more logical in terms of acceleration of data accumulation for risk assessment.
- Search for outcomes potentially useful for epidemiological research should be one of objectives at the designing of experiments with animals. There is increasing demand for this information to design human studies.

Finally we can conclude that at least one cause of the delay in decision making lies in frames of toxicological science. This cause consists in poor integration of human and experimental branches of toxicology. Hence, broadening of dialog between different branches of toxicology is needed.

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Table 1. Effects of low dose toxicity of BDE-47 in rat and sheep developmental models.

Dose, mg/kg bw	Effects	
	Rat model	Sheep model
0,0002	Not tested	Decreased total triiodothyronine
0,002	Hyperactivity, decreased anxiety, decreased total thyroxine, decreased corticosterone, impaired glucose metabolism.	Decreased total triiodothyronine and total thyroxine
0,02	Increased body weight and size, hyperactivity, decreased anxiety, decreased total thyroxine, decreased corticosterone, impaired glucose metabolism, decreased anogenital distance in mail offspring.	Decreased total thyroxine
0,2	Increased body weight and size, hyperactivity, decreased anxiety, decreased free and total thyroxine, decreased corticosterone, adrenal atrophy, impaired adrenal zonation, impaired expression of steroidogenic enzymes, increased cholesterol, impaired glucose metabolism, decreased anogenital distance and plasma IGF-1 in mail offspring.	Not tested

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Figure 1. Temporal distribution of studies addressing PCB (A) and PBDE (B) toxicity in experiments with animal models.

Figure 2. Temporal trends toward decrease of average daily dose in experiments with animal models addressing PCB (A) and PBDE (B) toxicity.

Figure 3. Distribution of animal species in experiments addressing PCB (A, C) and PBDE (B, D) toxicity: A, B – big taxonomic groups, C, D – mammalian models.

Figure 4. Distribution of endpoints of PCB and PBDE toxicity addressed in experiments with animal models.

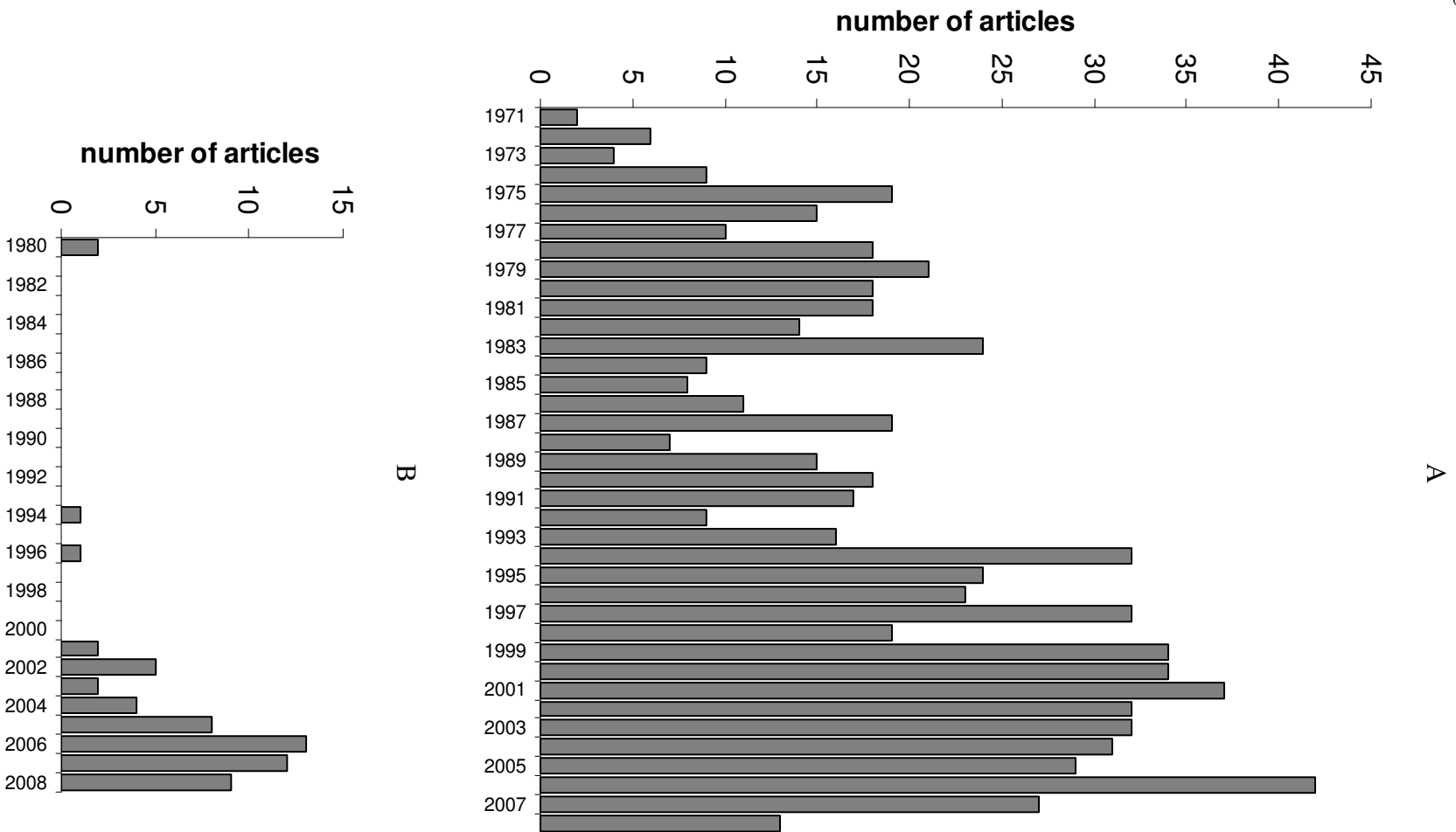
Figure 5. Distribution of endocrine endpoints of PCB (A) and PBDE (B) toxicity addressed in experiments with animal models.

Figure 6. Trends towards increase in number of developmental experiments *versus* experiments with adult animals addressing PCB (A) and PBDE (B) toxicity and increase in number of pre- and perinatal versus neonatal exposures within developmental experiments with PCB (C).

Figure 7. Temporal trends towards alteration in number of studies of PCB (A) and PBDE (B) toxicity which use estimation of internal dose.

Figure 8. Temporal trend towards increase in number of exposures *via* injections *versus* oral exposure in experiments with animal models addressing PCB toxicity.

Figure 1



A

B

Figure 2

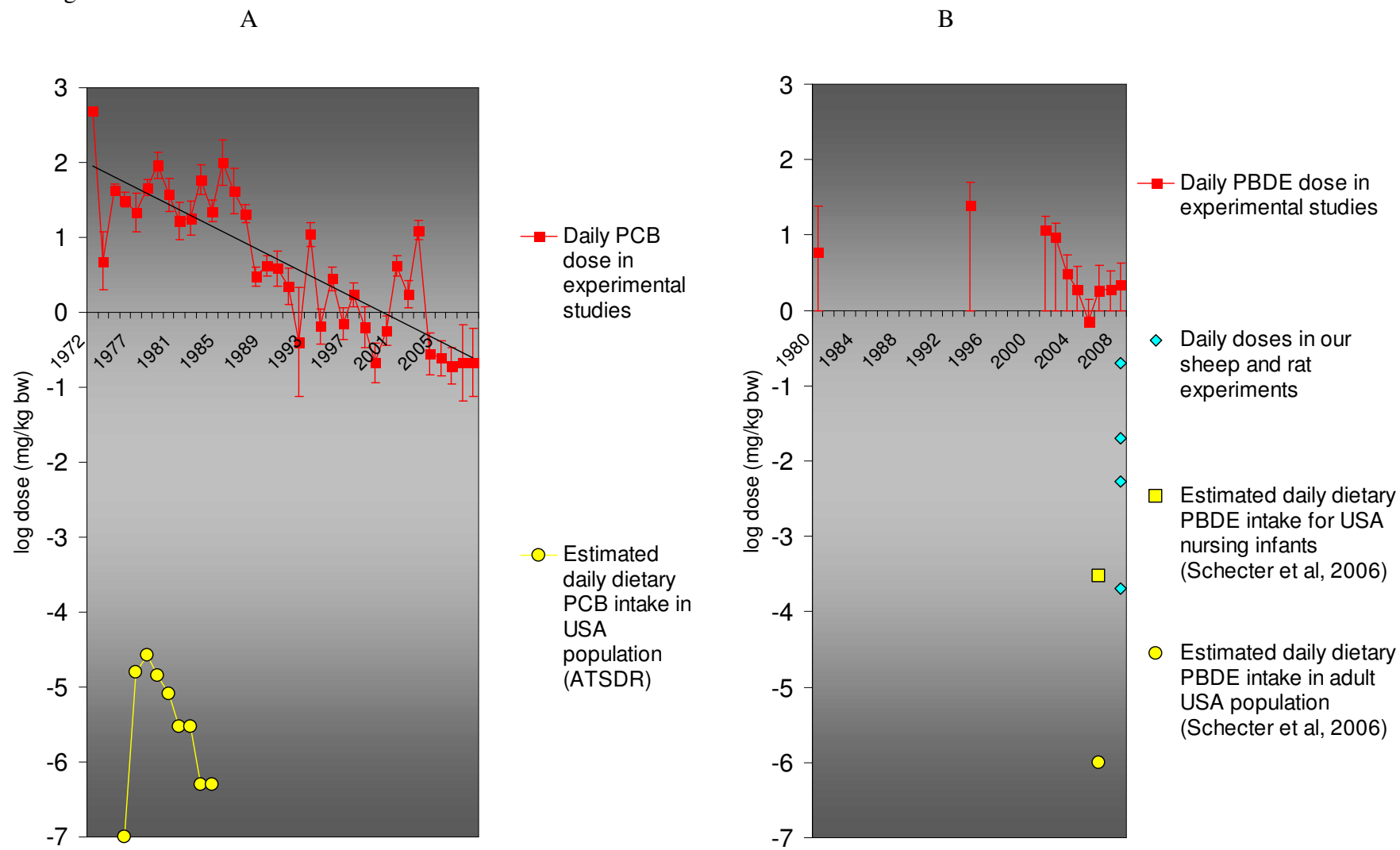


Figure 3

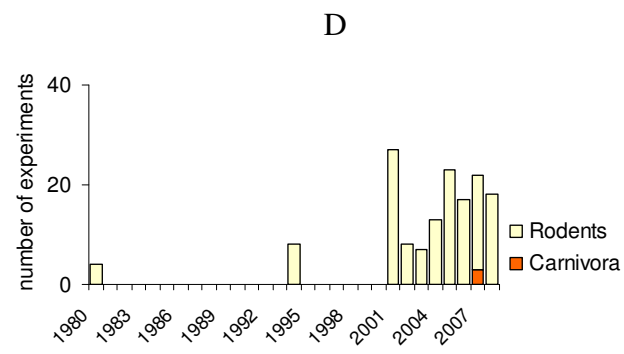
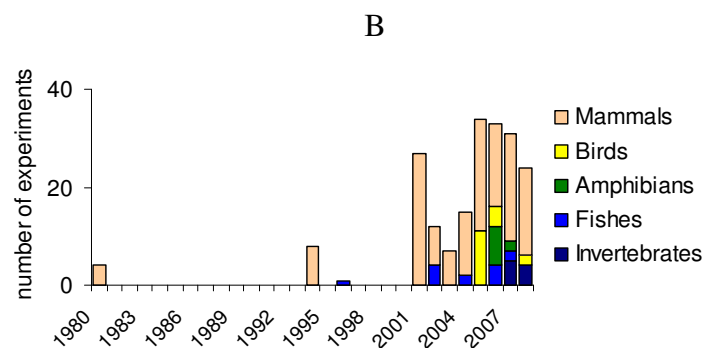
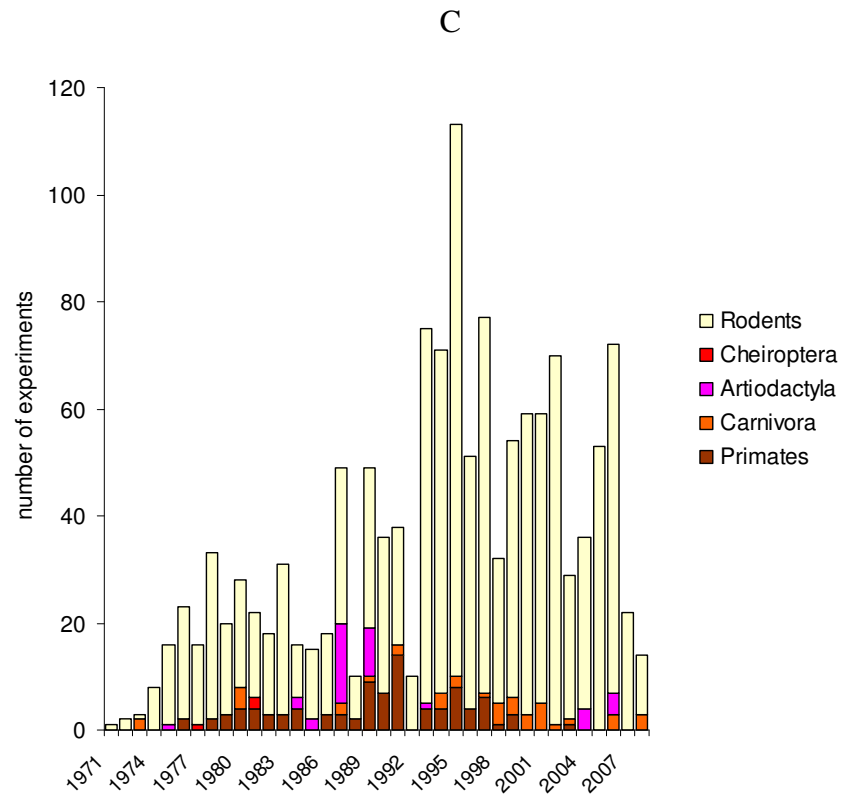
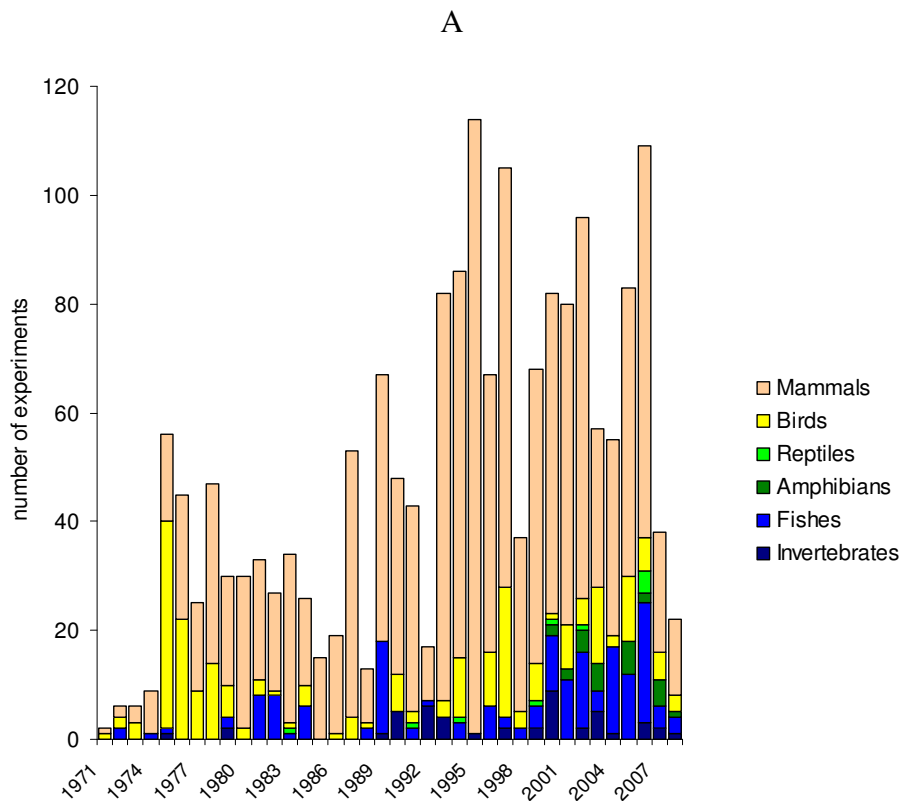


Figure 4

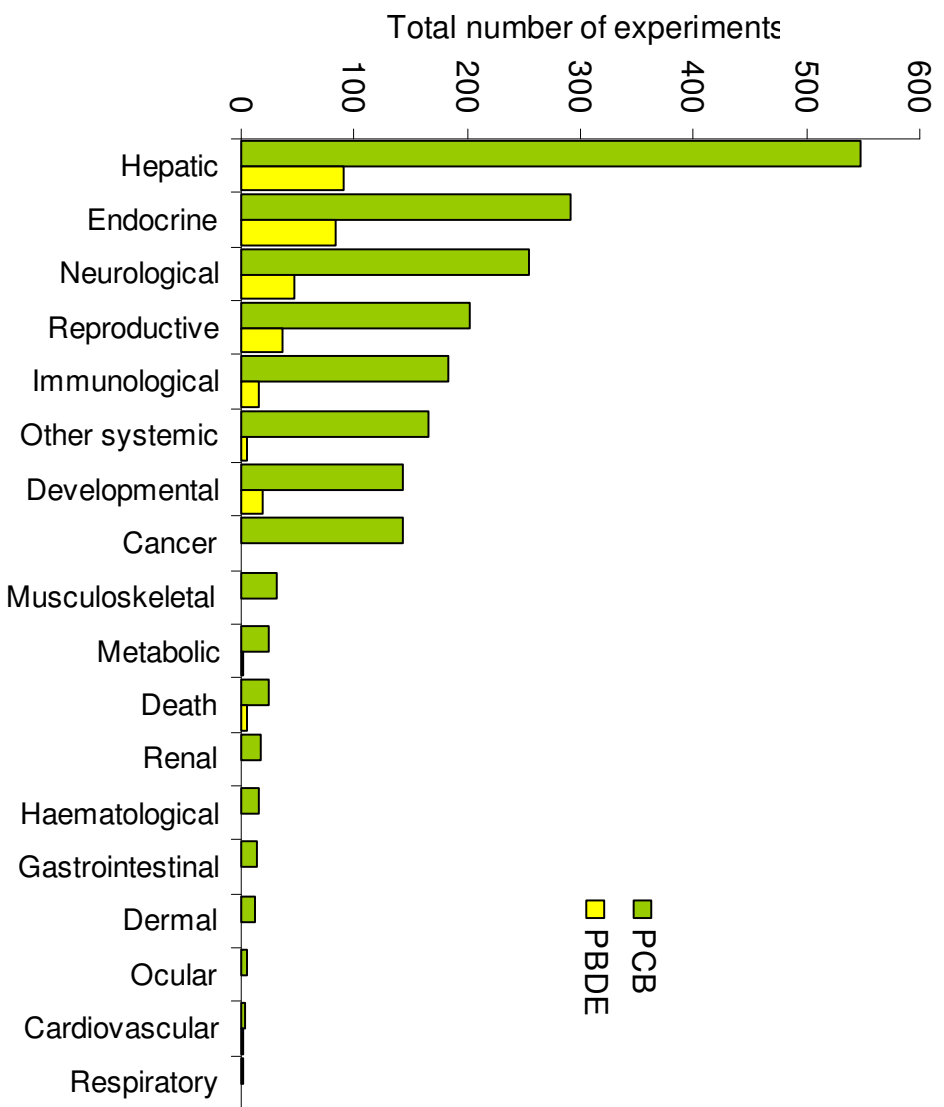
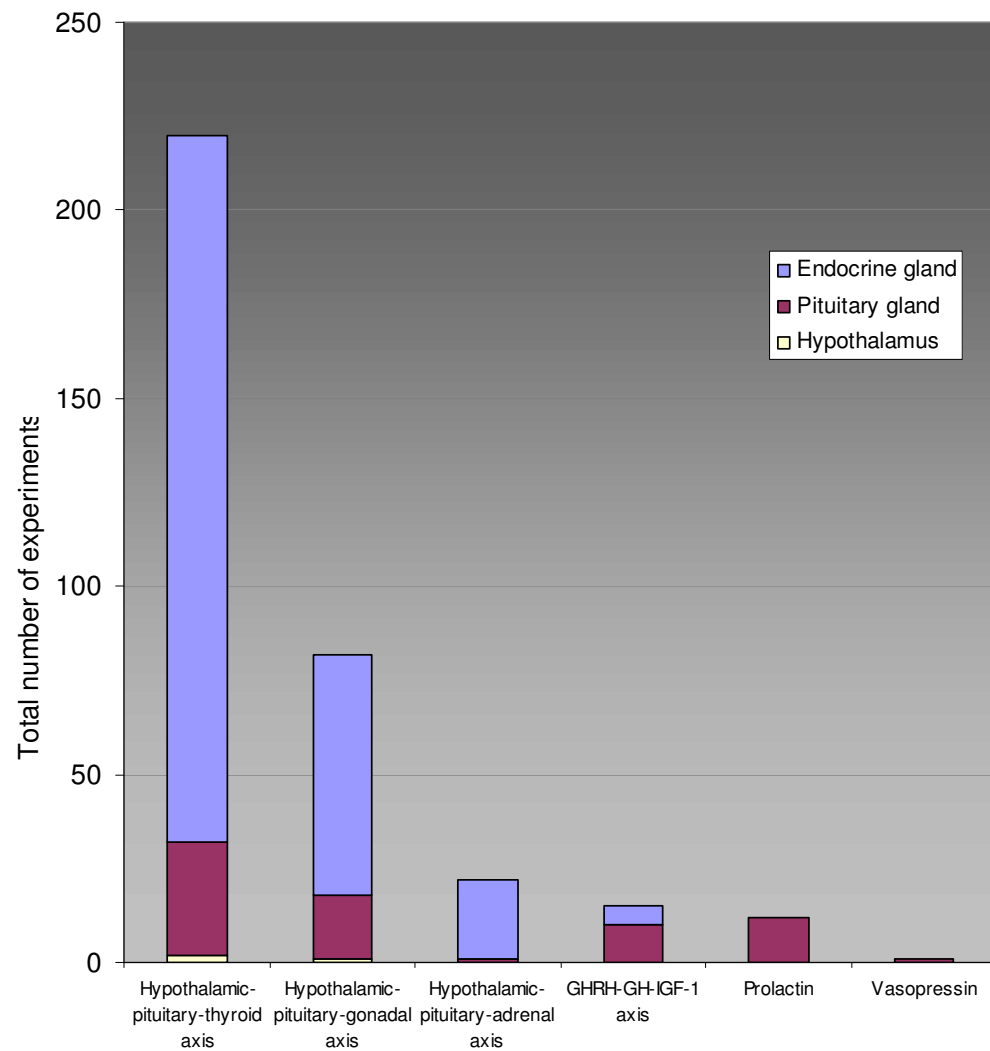


Figure 5

A



B

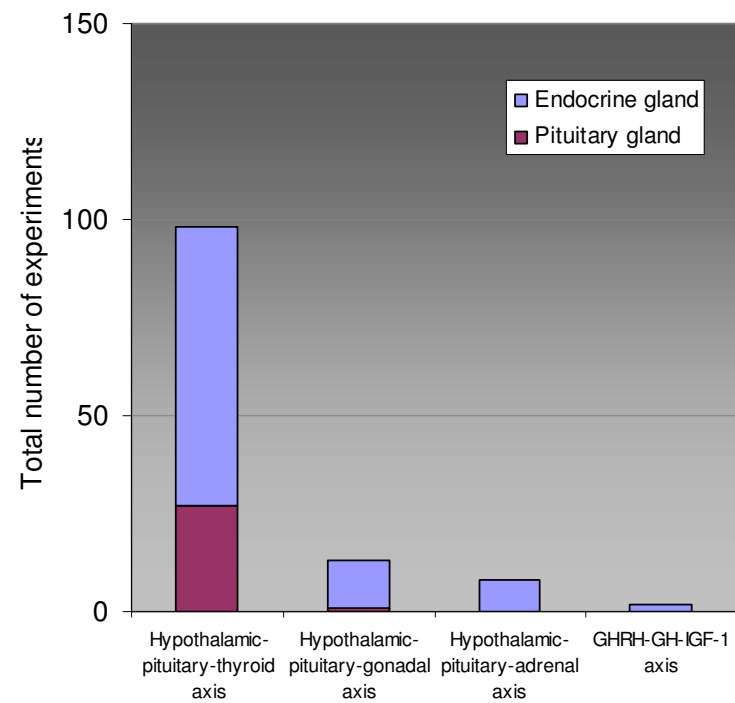
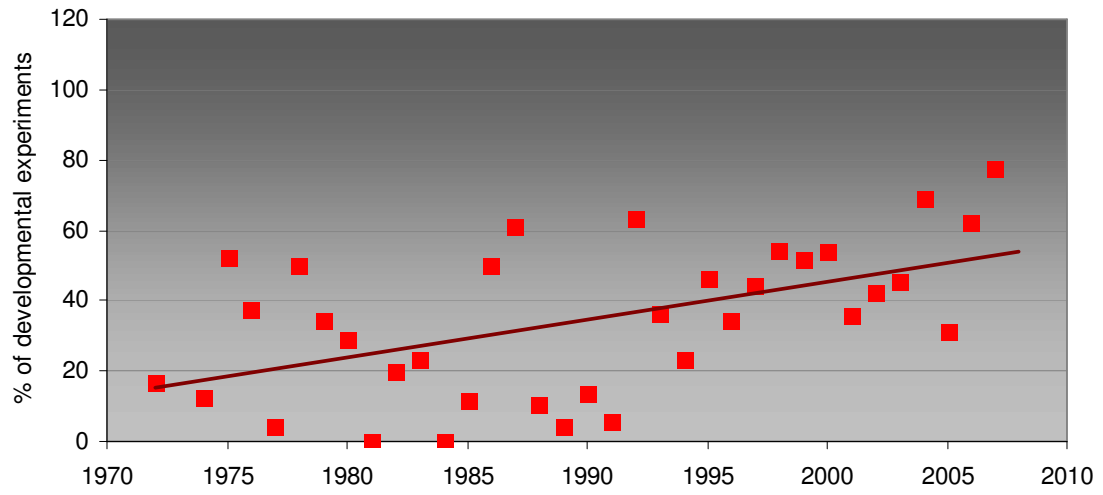
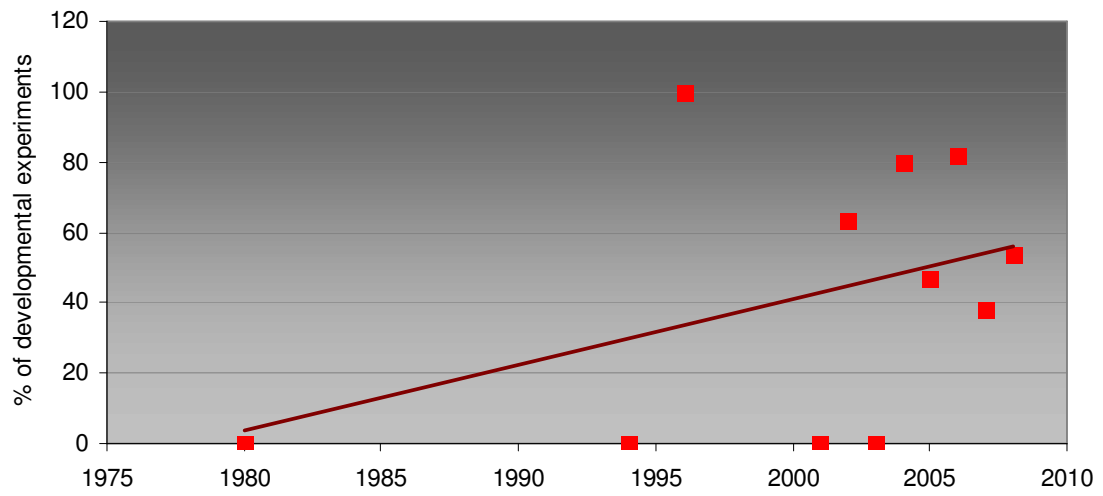


Figure 6

A



B



C

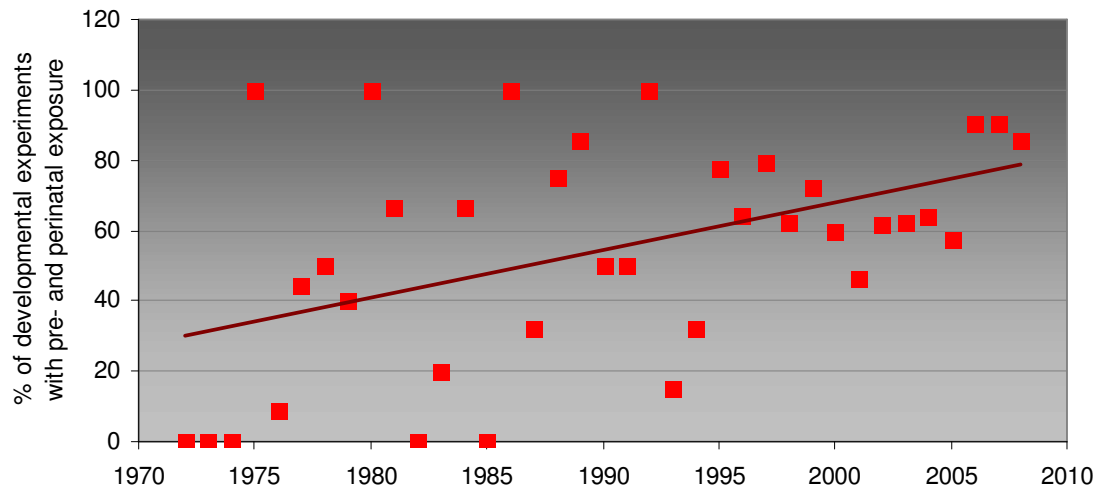
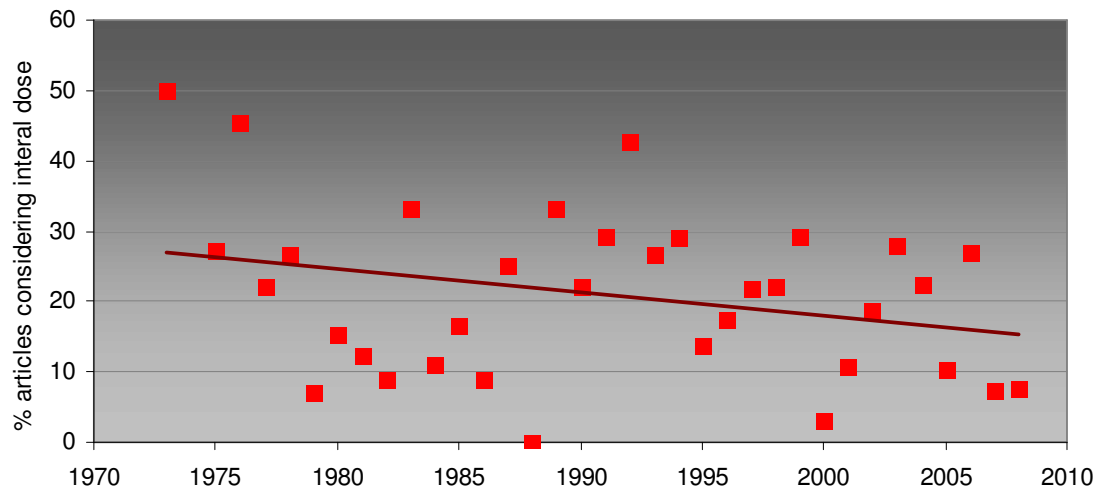


Figure 7

A



B

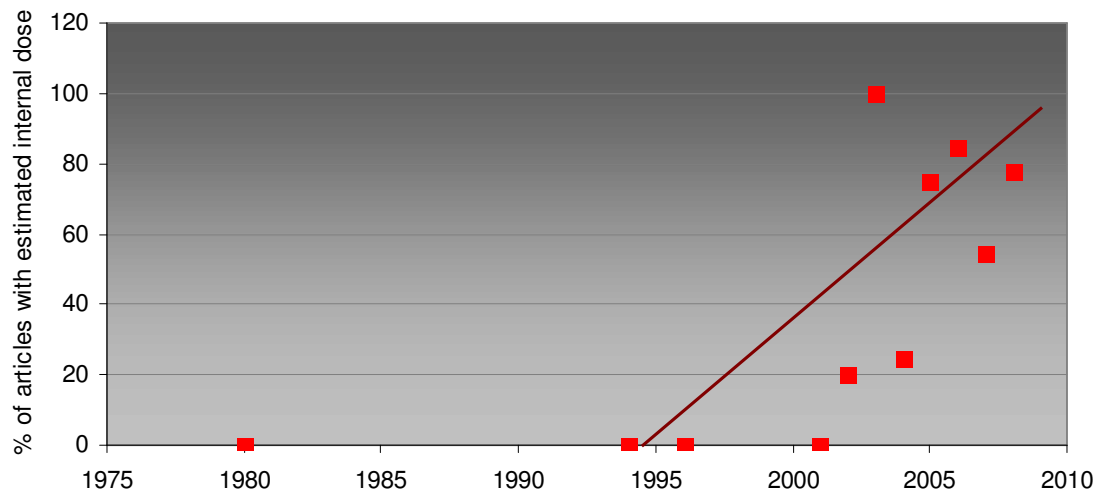


Figure 8

