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Arsenic-containing hydrocarbons are toxic in the in vivo model Drosophila melanogaster

Arsenic-containing hydrocarbons are toxic in the in vivo model *Drosophila melanogaster*†

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Arsenic-containing hydrocarbons (AsHC) constitute one group of arsenolipids that have been identified in seafood. In this first *in vivo* toxicity study for AsHCs, we show that AsHCs exert toxic effects in *Drosophila melanogaster* in a concentration range similar to that of arsenite. In contrast to arsenite, however, AsHCs cause developmental toxicity in the late developmental stages of *Drosophila melanogaster*. This work illustrates the need for a full characterisation of the toxicity of AsHCs in experimental animals to finally assess the risk to human health related to the presence of arsenolipids in seafood.

Inorganic arsenic (iAs) is classified as a human carcinogen. Additionally, chronic ingestion of iAs in humans has been associated with skin lesions, neurotoxicity, cardiovascular diseases, abnormal glucose metabolism, diabetes as well as a disturbance of foetal and infant development.[1] In 2009 the EFSA Panel on Contaminants in the Food Chain carried out a risk characterisation for arsenic in food and concluded that risks to human health related to the presence of inorganic arsenic cannot be excluded.[2] In the case of arsenosugars and arsenolipids, which occur mainly in seafood, no risk characterisation exists because of a lack of toxicological data. However, when arsenolipids are ingested by humans, they are bioavailable and can be efficiently metabolised.[3,4]

This class of fat-soluble arsenicals can be separated into arsenosugar-phospholipids (AsPL),[5] arsenic-containing fatty acids (AsFA),[6] the recently identified cationic trimethylarsonio

fatty alcohols (TMAsFOH)[7] and the arsenic-containing hydrocarbons (AsHC).[8]

AsHCs consist of a polar dimethylarsinoyl group and a long hydrocarbon chain, which is important for the lipophilic character of these compounds. AsHCs have been identified in edible fish oils,[7–9] several fish meat samples, including herring,[10] tuna[11] and cod[12,13] and also in some edible brown algae.[5]

Recently, three AsHCs (Fig. 1) have been shown to exert toxic effects in cultured human urothelial and liver cells with IC_{50} values similar to that of arsenite (iAs^{III}). However, the toxic modes of action seem to differ between iAs^{III} and the AsHCs. Because of their lipophilic properties, the AsHCs strongly accumulated in the cells, where, in contrast to arsenite, they strongly disturbed the cellular energy level.[14]

In the present study we further characterised the toxicological profiles of the three AsHCs (Fig. 1) by investigating their effects on an entire and intact organism. For the first model organism, we chose the common fruit fly *Drosophila melanogaster* because it is a well-characterised *in vivo* model exhibiting a high degree of homology to mammalian genes.[15] Further advantages of *Drosophila melanogaster* over other animals are that they are easy and fast to breed, and thus a whole life cycle from the parental flies over the larval and pupal stages to flies of the F1
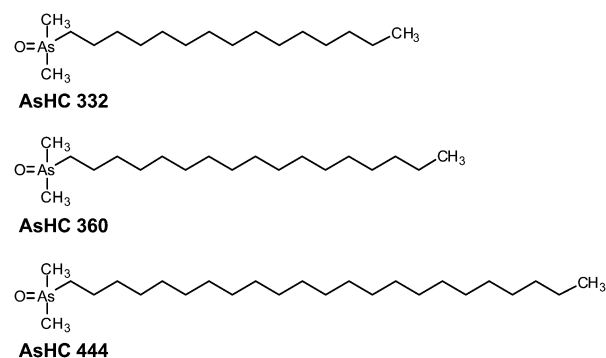
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Fig. 1 Chemical structures and abbreviations of three arsenic-containing hydrocarbons investigated in this study.

generation can be studied in a few weeks. For these reasons, *Drosophila melanogaster* is frequently applied as an *in vivo* model to screen compounds for their toxicity to get a first insight into their toxic modes of action.[16]

As toxicity markers, we assessed the survival of the parental flies, the resulting number of pupae as well as the eclosion, which describes the number of hatching adult flies (F1 generation) from the pupal stage. These lethality endpoints are eminently suitable for an efficient toxicological screening.[16] Effects of the three AsHCs were studied in direct comparison to the toxic reference arsenical iAs[III]. All crosses of wild type *Drosophila melanogaster* (OregonR kindly provided by C. Klaembt, Muenster, Germany) were performed at 25 °C and raised on *Drosophila melanogaster* medium (Formula 4-21, Carolina Biological Supply Company, Burlington, USA). Media were incubated with the stock solutions of the investigated arsenicals, yielding final concentrations of 50 to 200 μM. iAs[III] (>99.0%, Fluka Biochemika, Buchs, Germany) stock solutions were always freshly prepared in bi-distilled water before each experiment. AsHC 332, AsHC 360 and AsHC 444 were synthesised and purified (purity >99%) as described elsewhere.[17] Stock solutions (10 mM) of the AsHCs were prepared in 100% EtOH and stored at 4 °C. For incubation of media, the stock solutions were diluted shortly before the experiment with EtOH. The final EtOH concentration in the *Drosophila melanogaster* media (incubated with the AsHCs) was 2% in all experiments. An identical experiment incubated with 2% of EtOH, but without an arsenical, was used as reference. This EtOH concentration did not induce any significant effects compared to untreated control experiments (data not shown).

To start the experiment two male and three female flies were placed in each vial. On day three of the experiment the parental generation was removed. The third instar larval and pupal stage as well as the adult F1 generation flies were collected at the end of the experiment. For collecting, the flies were anaesthetized with CO_2 and stored at −20 °C until analysis (see below). For toxicity testing, pupae were marked on day 14 and the number of hatched flies was counted 7 days later to determine the rate of eclosion (Fig. 2).

In the tested incubation range, none of the arsenicals affected the survival of the parenteral flies (data not shown).
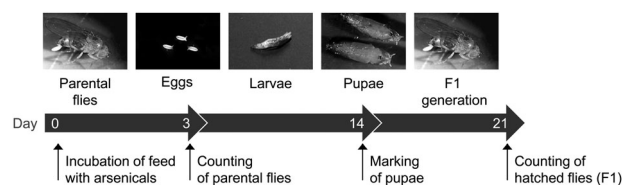

Fig. 2 Experimental set up for toxicity testing and representative pictures of the developmental stages of *Drosophila melanogaster*.
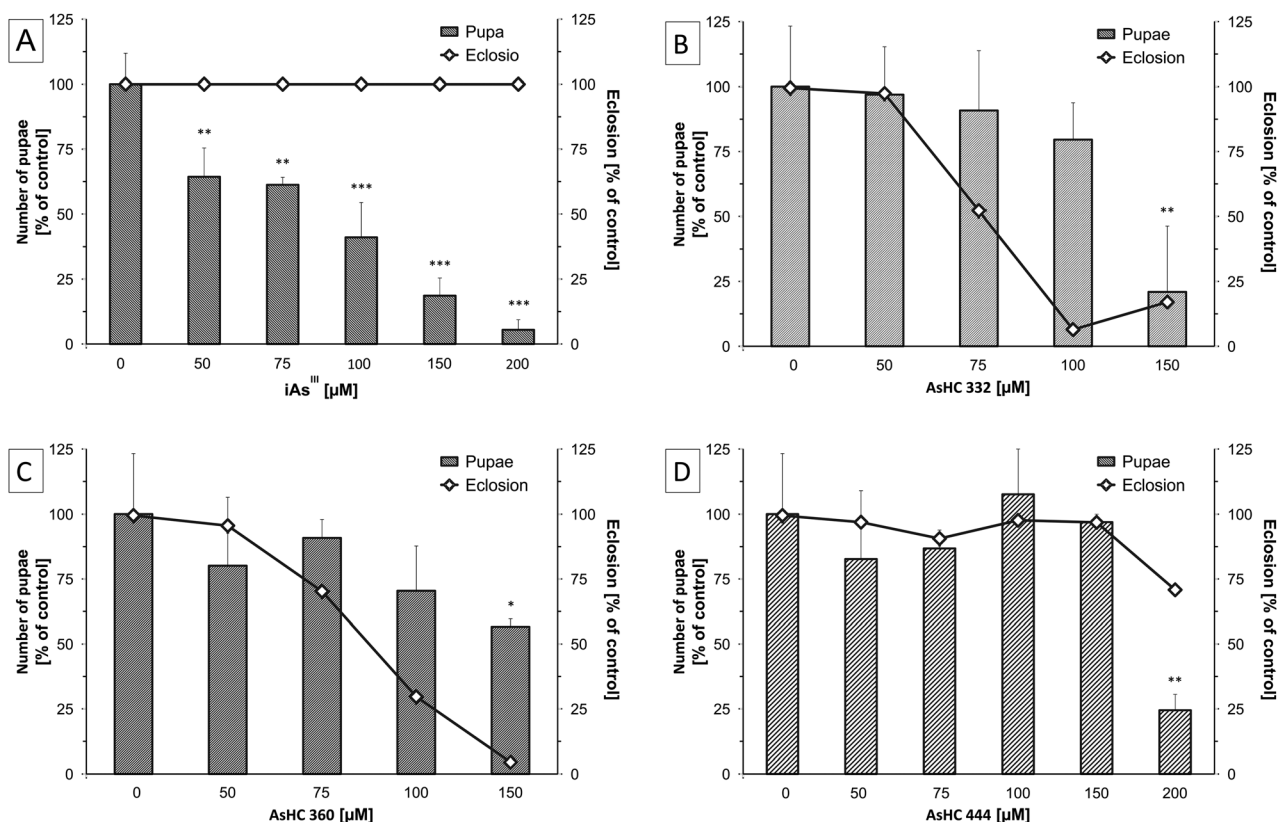

Fig. 3 Effects of iAs[III] (A), AsHC 332 (B), AsHC 360 (C) and AsHC 444 (D) containing media on the development of *Drosophila melanogaster*. Bars represent the number of pupae produced by two male and three female flies after 14 d of incubation normalised to a seeding on arsenic-free food (100% = 46.4 ± 9.7). Lines show percent adult eclosion 7 d after. Shown are mean values of at least three independent determinations (two vials each) +SD (ANOVA-OneWay-test; significance levels: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$).
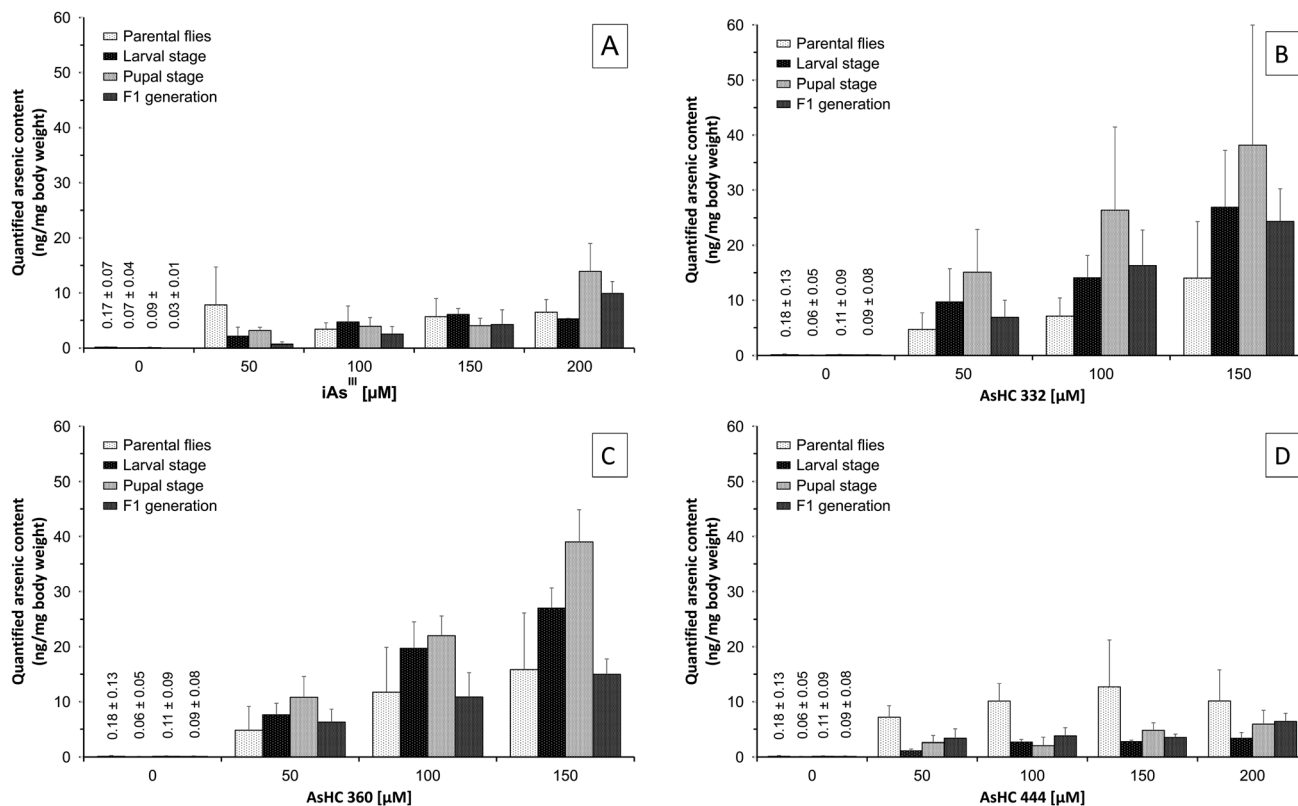
Fig. 4 Arsenic content of the distinct stages of *Drosophila melanogaster* after exposure to iAs[III] (A) and AsHC 332 (B), AsHC 360 (C) and AsHC 444 (D) by media determined by ICP-MS/MS. Neither of the arsenicals affected the body weight of parenteral flies (0.77 ± 0.25 mg), larvae (1.16 ± 0.56 mg), pupae (1.00 ± 0.29 mg) and flies of the F1 generation (0.68 ± 0.25 mg). The figure represents the mean values of at least three independent determinations (two vials each) +SD.

However, all three AsHCs and iAs[III] showed significant effects on the development of *Drosophila melanogaster*. In the case of iAs[III] a concentration-dependent reduction in the number of pupae was observed, with significant effects occurring already at 50 µM iAs[III] (Fig. 3A). Nevertheless, iAs[III] had no influence on the late development cycle of *Drosophila melanogaster* from the larval over the pupal stage to hatched flies of the F1 generation. Pupae of incubated parental flies were still able to pupate and hatch, albeit less successfully compared to untreated flies (Fig. 3A). No dead larvae were visible in the vials. These findings match results from a previous study[18] with *Drosophila melanogaster* exposed to iAs[III] for 7 days, where a LC$_{50}$ of 540 µM was found and 250 µM iAs[III] was not toxic to parental flies, but induced a yield reduction of about 60%. These results indicate that iAs[III] interrupts the life circle or development of *Drosophila melanogaster* at an early stage. Thereby iAs[III] might affect not only the laying of eggs but also disturbs the development from the embryonal to the early larval stage, an effect that has been postulated before.[19]

The effects for the AsHCs were different from those observed for iAs[III]. All three AsHCs decreased the number of pupae only in the highest incubation concentration, but in contrast to arsenite they disturbed the hatching of flies from pupae (Fig. 3B–D). These data indicate that these three arsenolipids exert an impact on the late developmental stages of *Drosophila melanogaster*. AsHC 332 and AsHC 360 nearly completely inhibited the hatching of flies (F1 generation) from the pupal stage. Interestingly, the most lipophilic arsenolipid investigated, AsHC 444, exerted the lowest developmental toxicity in *Drosophila melanogaster*. This fits nicely to the *in vitro* studies, where AsHC 444 was less toxic than both AsHC 332 and AsHC 360. In liver cells, the lower toxicity of AsHC 444 correlated well with its lower cellular bioavailability.[14]

To study the bioavailability of arsenite and the arsenic-containing hydrocarbons in *Drosophila melanogaster* as well, the total arsenic contents in samples of the different developmental stages of *Drosophila melanogaster* were determined by inductively coupled plasma mass spectrometry (ICP-MS/MS, Agilent ICP-QQQ 8800, Waldbronn, Germany). Incubated flies of the parental, larval and pupal stage as well as flies of the F1 generation were washed three times with PBS and digested with a mixture of 65% HNO$_3$ and 30% H$_2$O$_2$ (1 : 1) for at least 12 h at 95 °C to dryness. The residue was dissolved and diluted with 0.15 M HNO$_3$ for ICP-MS/MS measurement. Arsenic was quantified in the MS/MS mode using oxygen as reaction gas as described before (for ICP-MS parameters see ESI†).[14]

For all arsenicals, the arsenic content of the *Drosophila melanogaster* samples increased with increasing exposure concentration, although there were differences between the arsenicals. Thus, after incubation with iAs[III] the arsenic content in all stages of development of *Drosophila melanogaster* was
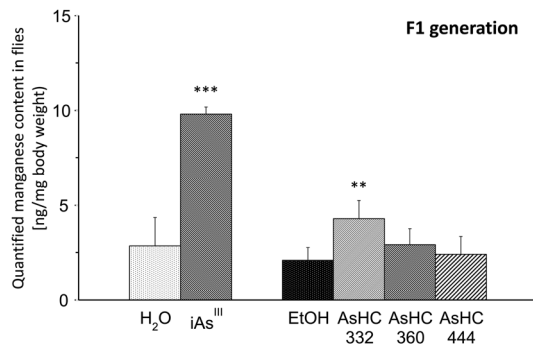
Fig. 5 Manganese content in flies of the F1 generation after exposure to 100 μM of iAs[III], AsHC 332, AsHC 360 and AsHC 444 by media determined by ICP-MS. The figure represents the mean values of two independent determinations (two vials each) +SD (ANOVA-OneWay-test; significance levels: **$p < 0.01$ and ***$p < 0.001$).

between 2–14 ng mg^{-1} body weight (Fig. 4A), whereas after incubation with AsHC 332 or 360, the total arsenic content was higher, especially in the larvae and pupae, ranging between 8–40 ng mg^{-1} body weight (Fig. 4B and C). AsHC 444 produced different results: it exerted the lowest bioavailability of the investigated arsenolipids (Fig. 4D), and in contrast to the other arsenicals studied, arsenic contents in larvae, pupae and flies of the F1 generation were always lower compared to arsenic contents in parenteral flies after feeding with AsHC 444. This lower bioavailability of AsHC 444 in larvae, pupae and flies of the F1 generation might be one reason for the less pronounced developmental toxicity of this arsenical. Interestingly, applied arsenic content of media (20–80 mg kg^{-1}) as well as the determined total arsenic content in all developmental stages of *Drosophila melanogaster* (2–40 mg kg^{-1}) were in the area of arsenic contents in commercial fish oils and certain organs of marine fish (1–19 mg kg^{-1}).[20]

In the next step, we quantified the content of the essential trace elements manganese, iron, copper and zinc in all developmental stages of *Drosophila melanogaster* after incubation of media each with 100 μM of the respective arsenicals (for ICP-MS parameters see ESI†). Neither arsenite nor the AsHCs affected the manganese, iron, copper or zinc content of parenteral flies, larvae or pupae (data not shown). Likewise, the contents of iron, copper and zinc were not disturbed by the arsenicals in flies of the F1 generation (data not shown). Nevertheless, arsenite and AsHC 332 significantly increased the manganese content in flies of the F1 generation (Fig. 5). The mechanisms behind as well as the consequences of the disturbed manganese homeostasis in the flies of the F1 generation are unclear and should be further investigated.

Conclusions

In this first *in vivo* toxicity study with *Drosophila melanogaster*, arsenic-containing hydrocarbons showed toxicity in a concentration range similar to that for arsenite. These results illustrate the need for a full characterisation of the toxicity of arsenic-containing hydrocarbons in experimental mammals in order to assess the risk to human health related to the presence of arsenolipids in seafood.

Acknowledgements

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