Wild birds as a bioindicator for wildlife toxicity in pineapple cultivation areas in Northern Costa Rica
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To my family and my husband Rodolfo
Contents

Contents................................................................................................................................................ IV
List of tables ........................................................................................................................................... VIII
List of figures ......................................................................................................................................... IX
Abbreviations ........................................................................................................................................ X
1.  Introduction ................................................................................................................................... 13
    1.1.  Background .......................................................................................................................... 13

1.2.  Pesticides ................................................................................................................................... 15

    1.2.1 Bromacil ............................................................................................................................ 15

    1.2.2 Diuron .................................................................................................................................. 16

    1.2.3 Ametryn ........................................................................................................................... 16

    1.2.4 Hexazinone ....................................................................................................................... 17

    1.2.5 Glyphosate ....................................................................................................................... 17

    1.2.6 Diazinon ........................................................................................................................... 18

    1.2.7 Carbaryl ........................................................................................................................... 19

    1.2.8 Hydramethylnon .............................................................................................................. 19

    1.2.9 Fosetyl-Aluminum .......................................................................................................... 20

    1.2.10 Metalaxyl ....................................................................................................................... 21

    1.2.11 Ethoprophos ................................................................................................................... 21
1.2.12 Oxamyl ........................................................................................................................ 22
1.2.13 Chlorfluorenol-Methyl .................................................................................................. 22
1.2.14 Ethylene ....................................................................................................................... 23

1.3. The effect of organophosphates and carbamates on cholinesterase activity ................. 23
1.3.1 Butyrylcholinesterase .................................................................................................... 24
1.3.2 Acetylcholinesterase ...................................................................................................... 24
1.3.3 Inhibition mechanism .................................................................................................... 26
1.3.4 Secondary effects in birds ............................................................................................. 26

   A) Acute Toxicity .......................................................................................................... 27
   B) Sublethal Toxicity .................................................................................................... 28
   C) Effects on feeding behavior ...................................................................................... 28
   D) Effects on the endocrine system and reproductive behavior ................................... 29
   E) Effect on thermoregulation ....................................................................................... 30
   F) Effect on the hematological system and immune system response ......................... 30

1.4. Wild birds as biomonitors for environmental contamination ........................................ 31
1.5. Pesticide contamination in feather and tissue samples ................................................... 32
1.6. Wild bird species ........................................................................................................... 33
1.7. Pesticide analysis with the QuEChERS method ............................................................. 34

2. Objectives .......................................................................................................................... 36
2.1. General objective ....................................................................................................................... 36

2.2. Specific objectives ..................................................................................................................... 36

3. Materials and methods ..................................................................................................................... 37

3.1. Enzyme activity measurements ................................................................................................. 37

3.1.1 Sample collection ................................................................................................................. 37

3.1.2 Plasma cholinesterase activity measurements ...................................................................... 40

3.1.3 Acetylcholinesterase activity measurements in brain tissue .............................................. 41

3.1.4 In vitro inhibition of butyrylcholinesterase by carbaryl ...................................................... 44

3.2. Sample collection for pesticide contamination and extraction method ..................................... 45

3.2.1 Feathers .............................................................................................................................. 46

3.2.2 Skin and feces ..................................................................................................................... 47

3.2.3 Soil .................................................................................................................................. 48

3.3. Pesticide Analysis by UHPLC-TOF-MC .................................................................................. 48

3.4. Statistical model ...................................................................................................................... 53

4. Results ........................................................................................................................................ 54

4.1. Environmental observations .................................................................................................. 54

4.2. Enzyme activity ...................................................................................................................... 57
4.2.1 Plasma butyrylcholinesterase activity ................................................................. 57
4.2.2 Acetylcholinesterase activity in brain tissue ......................................................... 64
4.2.3 In vitro inhibition of butyrylcholinesterase activity by carbaryl in plasma from control parrots ................................................................................................................. 70
4.3. Pesticides .................................................................................................................. 70

Feathers ....................................................................................................................... 70
Skin ............................................................................................................................... 73
Feces ............................................................................................................................. 73
Soil .............................................................................................................................. 73
5 Discussion .................................................................................................................. 74
6 Conclusions ............................................................................................................... 83
7 Outlook ..................................................................................................................... 84
8 Summary .................................................................................................................... 86
9 Zusammenfassung ...................................................................................................... 88
10 References ............................................................................................................... 90
11 Acknowledgement ................................................................................................. 143
List of tables

Table 1. List of the most frequently used pesticides in the pineapple-growing area in Northern Costa Rica (Ricart-Ballarà et al., 2009) ........................................................................................................14

Table 2. Chromatographic conditions regarding the used percentage of the mobile phases A and B, the flows and the retention times ........................................................................................................50

Table 3. Parameters used in the UHPLC and flight mass spectrometer ...........................................................51

Table 4. Calibration curves according to the analyzed pesticides ....................................................................52

Table 5. Descriptive statistics of plasma BChE activity (IU/L) measured in free-living wild birds by species in conventional and organic pineapple-growing areas. ........................................58

Table 6. Descriptive statistics of brain AChE activity (IU/g tissue) measured in free-living wild birds in the conventional and organic pineapple-growing areas ........................................................................65

Table 7. Results of the in vitro test: inhibition of BChE in plasma of control parrots by carbaryl. ....70

Table 8. Amount of diuron and ametryn (mg/kg) found in each analyzed sample by gender and species ..................................................................................................................................................72

Table 9. Summary of the pesticides detected in the soil samples collected in the conventional pineapple-growing areas. ............................................................................................................................................73
List of figures

Figure 1. A) Regional map of Costa Rica indicating the collecting area (black square). B) Map of the pineapple plantations in Northern Costa Rica (green zones indicate the organic lots and the yellow ones the conventional lots; white points mark organic sample points and black points mark conventional ones). Source: Soil and More International, Dole Food Company (2011)...........39

Figure 2. Sample point in a conventional pineapple-growing area: Mist nets were installed at the border between the pineapple plantation areas and the surrounding gallery forests with water sources.................................................................................................................................................40

Figure 3. Application of agrochemicals in a conventional pineapple area. ........................................54

Figure 4. Agrochemical application board in a conventional pineapple area. ...................................55

Figure 5. View of an organic pineapple area. ........................................................................................56

Figure 6. A Sporophila americana with a Ramphocelus passerinii in a conventional pineapple zone. .....................................................................................................................................................57

Figure 7. Butyrylcholinesterase (BChE) activity of all samples by pineapple cultivation area...........59

Figure 8. Butyrylcholinesterase (BChE) activity per species in each growing area. .........................61

Figure 9. Butyrylcholinesterase (BChE) activity in serum by gender, sampled species and the pineapple-growing areas. .....................................................................................................................................................63

Figure 10. Acetylcholinesterase (AChE) values of all samples by pineapple cultivation areas. ........65

Figure 11. Acetylcholinesterase (AChE) activity per species in each sampled growing area..........67

Figure 12. Acetylcholinesterase (AChE) activity (IU/g) in brain tissue by gender, sampled species and the pineapple-growing area. Differences were considered as significant at a level of p ≤ 0.05 (*). ........................................................................................................................................................69
Abbreviations

°C Degrees Celsius
µg Microgram
µl Microliter
µs Microsecond
ACh Acetylcholine
AChE Acetylcholinesterase
Anti-ChE Anti-Cholinesterase
BCh Butyrylcholine
BChE Butyrylcholinesterase
BSA Bovine Serum Albumin
C18 Octadecylsilan
CI Confidence Interval (of the mean)
DNA Deoxyribonucleic Acid
e.g. Exempli gratia
ELISA Enzyme-Linked Immunosorbent Assay
ESI Electro-Spray Ionization
EU European Union
eV Electron Volts
g Gram
GC Gas Chromatography
HPLC High Performance Liquid Chromatography
i.e. Id est
iPrOH  Isopropyl Alcohol
kg    Kilogram
kHz   Kilohertz
LC₅₀  Median Lethal Concentration
LC    Liquid Chromatography
LD₅₀  Median Lethal Dose
LH    Luteinizing Hormone
LOAEL Lowest Observed Adverse Effect Level
LOC   Level of Concern
LOD   Limit of Detection
LOQ   Limit of Quantification
MeCN  Acetonitrile
mg    Milligram
mM    Molar Mass
MS    Mass Spectrometry
m/z   Mass to charge ratio
nm    Nanometer
nmol  Nanomole (10⁻⁹)
NOEC  No Observable Effect Concentration
OCPs  Organochlorine Pesticides
PCBs  Polychlorinated Biphenyls
pH    Potential of Hydrogen Scale
POPs  Persistent Organic Pollutants
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSA</td>
<td>Primary Secondary Amine</td>
</tr>
<tr>
<td>QuEChERS</td>
<td>Quick, Easy, Cheap, Effective, Rugged and Safe</td>
</tr>
<tr>
<td>S.E.M.</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>SLE</td>
<td>Solid Liquid Extraction</td>
</tr>
<tr>
<td>S/N</td>
<td>Signal-to-noise Ratio</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid Phase Extraction</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of Flight</td>
</tr>
<tr>
<td>TPP</td>
<td>Triphenylphosphate</td>
</tr>
<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>UHPLC</td>
<td>Ultra High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>Vpp</td>
<td>Peak-to-peak Voltage</td>
</tr>
<tr>
<td>vs</td>
<td>versus</td>
</tr>
</tbody>
</table>
1. Introduction

1.1. Background

According to the Food and Agriculture Organization of the United Nations, the pineapple production has increased worldwide more than threefold in the last 30 years. After banana and citrus, pineapple production is the most important tropical fruit crop. Eighty five percent of the imported pineapples in the United States of America and Canada as well as seventy one percent in Europe come from Costa Rica (FAO, 2011). Costa Rica is one of the worldwide biggest producers of tropical fruits, and has recently become the world's largest exporter of pineapples (RAMIREZ et al., 2009; BRAVO et al., 2011). The use of pesticides is a common way to control agricultural pests and this Central American country has therefore also become the biggest user of pesticides in this region between 1977 and 2000, with 150 000 tons of active substances having been imported (VALCKE et al., 2005; BRAVO et al., 2011). Pineapple production uses an average of 30 kg of active pesticide ingredients per hectare per year, and currently crops are grown on a surface of 45 000 hectares (CASTILLO et al., 2012). In general, the trend towards an increased import and use of pesticides is due to a more intensive cultivation and an increase in the size of crop-growing areas. BRAVO et al. (2011) reported that, historically, fungicides (46%) have been the most commonly imported products, followed by herbicides (29%), insecticides-nematicides (16%) and fumigants (8%). While fungicides are used mainly on bananas, herbicides and nematicides are used on bananas as well as on pineapples (BRAVO, 2007; POLIDORO, 2008). The intensive field cultivation is related to a great amount of environmental and health-related concerns, for example: ecological disorders, soil erosion, pesticide contamination and
degradation of natural habitats, water sources and human exposure to agrochemicals (SANDOVAL, 2009).

The substances listed in Table 1 represent the most frequently used chemicals in the pineapple-growing areas according to RICHART-BALLARA et al. (2009).

**Table 1. List of the most frequently used pesticides in the pineapple-growing area in Northern Costa Rica (Ricart-Ballarà et al., 2009)**

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Chemical group</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bromacil</td>
<td>Pyrimidindione</td>
<td>Herbicide</td>
</tr>
<tr>
<td>Diuron</td>
<td>Phenyl-urea derivative</td>
<td>Herbicide</td>
</tr>
<tr>
<td>Ametryn</td>
<td>Triazine</td>
<td>Herbicide</td>
</tr>
<tr>
<td>Hexazinone</td>
<td>Triazine</td>
<td>Herbicide</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>Phosphonate</td>
<td>Herbicide</td>
</tr>
<tr>
<td>Diazinon</td>
<td>Organophosphate</td>
<td>Insecticide</td>
</tr>
<tr>
<td>Carbaryl</td>
<td>Carbamate</td>
<td>Insecticide</td>
</tr>
<tr>
<td>Hydramethylnon</td>
<td>Trifluoromethyl aminohydrazone</td>
<td>Insecticide</td>
</tr>
<tr>
<td>Fosetyl-Aluminum</td>
<td>Phosphonate</td>
<td>Fungicide</td>
</tr>
<tr>
<td>Metalaxyl</td>
<td>Acetylalanines</td>
<td>Fungicide</td>
</tr>
<tr>
<td>Ethoprophos</td>
<td>Organophosphate</td>
<td>Insecticide/Nematicide</td>
</tr>
<tr>
<td>Oxamyl</td>
<td>Carbamate</td>
<td>Insecticide/Nematicide</td>
</tr>
<tr>
<td>Chlorflurenol-Methyl</td>
<td>Morphactine</td>
<td>Plant growing factor</td>
</tr>
<tr>
<td>Ethylene</td>
<td>Hydrocarbon</td>
<td>Plant growing factor</td>
</tr>
</tbody>
</table>
Pesticides are widely used to combat diseases and pests, but they may also adversely affect the availability of vegetable and animal sources that are nutrients for other wild animal species. They comprise a large number of substances that chemically belong to many completely different classes and consequently have varying modes of action, uptake, biotransformation and elimination pathways. (PICO et al., 2003).

1.2. Pesticides

1.2.1 Bromacil

Bromacil is an herbicide, which inhibits photosynthesis in plants and belongs to the pyrimidindione group. Bromacil is reported as practically non toxic to wild birds. In vivo studies in bobwhite quails (Colinus virginianus) (LD$_{50}$ greater than 2250 mg/kg) showed that bromacil administered per os did not lead to toxicity. Some laboratory studies with bromacil reported oral intoxication in white leghorn chickens (Gallus gallus domesticus) given 500 mg/kg/day (PALMER u. RADELEFF, 1969) and in mallard ducks (Anas platyrhynchos) administered more than 10000 mg/kg/day (WASHINGTON STATE DEPARTMENT OF TRANSPORTATION, 2006). In both cases, the treated birds showed the same decreased body weight. It became highly toxic in the case of concentrations greater than 10 mg/kg in bobwhite quails and mallard ducks after feeding the substance for 8 days. There is limited information on its chronic toxicity in wild birds.

The half-life of this herbicide in soils is approximately 60 days, which could be extended to 8 months depending on the ambient conditions. It can contaminate underground water sources because it easily passes through soil and reaches the roots of the plant.
1.2.2 Diuron

Diuron is a phenylurea derivate and an herbicide, which inhibits plant photosynthesis (WESSELS u. VAN DER VEEN, 1956) and is used for the control of broadleaf and grassy weed. Diuron can also be slightly toxic to birds. The LC$_{50}$ for bobwhite quail fed diuron is 1730 mg/kg. However, an LC$_{50}$ greater than 5000 mg/kg for Japanese quail ($Coturnix japonica$), ring-necked pheasants ($Phasianus colchicus$) and mallard ducks indicates low toxicity in these bird species (WEED SCIENCE SOCIETY OF AMERICA, 1994).

Diuron can be detected in soils in the period of time from 30 to 365 days, mostly found 90 days after application (WAUCHOPE et al., 1992). It easily migrates to the roots and less efficiently to leaves and stems.

1.2.3 Ametryn

Ametryn is a triazine herbicide, which stops photosynthesis and also influences other enzymatic processes in plants. It is slightly toxic to large ($Anas platyrhynchos$) and small ($Colinus virginianus$) birds (LD$_{50}$ greater than 2250 mg/kg body weight as well as five-day-LC$_{50}$ greater than 5620 mg/kg) in the case of acute oral exposure (GRIMES AND JABER, 1988a). Reduced growth, poor weight gain and adverse reproduction effects were reported as effects due to chronic exposure to ametryn (BEAVERS, 1990).

The environmental behavior of ametryn depends on the kind of soil and its specific properties. It can be detected 10 to 38 days. Its degradation is due to aerobic soil metabolism, and in some cases, because of its stability, it can stay in soil for up to one year. In the pineapple cultivation areas, ametryn has mostly been detected in the short grasses (WONG, 1991).
1.2.4 Hexazinone

The herbicide hexazinone belongs to the triazine group and helps to control a broad range of weeds in sugar cane, pineapple and lucerne growing areas. Rainfall or irrigation water is needed before it becomes activated. It is persistent in soils and aquatic environments. It should not be used 30 to 60 days before grazing, harvest or feeding (USDA, 1984).

Hexazinone is nontoxic to birds in case of acute and subacute oral application (WEED SCIENCE SOCIETY OF AMERICA, 1994).

The pesticide can be detected in water sources six months after the last application. It can persist and be mobilized in soil and aquatic ecosystems. Therefore, hexazinone could be viewed as a possible concern for water contamination (USDA, 1984).

1.2.5 Glyphosate

Glyphosate, as a representative of the phosphonates, is a non-selective herbicide, which in low doses can be used as a growing regulator. It can cause eye or skin irritation during its preparation. The effects of this chemical in mammals, birds, fishes and invertebrates are reported to be minimal, only restricting its collateral adverse effects to some aquatic non-target plants (KIDD u JAMES, 1991).

Glyphosate is adsorbed to soil particles and its residues remain immobilized in the ground and can be decomposed by microbes (WAUCHOPE et al., 1992). The pesticide can persist in soil between seven and 900 days, depending on the kind of soil, local climate, frequency of the application and terrestrial field dissipation (WEED SCIENCE SOCIETY OF AMERICA, 1994).
1.2.6 Diazinon

Diazinon is an organophosphate (OP) used as insecticide, acaricide and nematicide that without mitigation can cause serious risks to agricultural workers and wildlife bird species. It is the one of the most widely used insecticides for agricultural pest control. Diazinon is also used in veterinary medicine against fleas and ticks. It is available as dust, granules, seed dressings, wet table powder, and emulsifiable solution formulations.

Diazinon is highly toxic to birds and has been linked to a number of bird deaths. The acute oral toxicity LD_{50} of diazinon, administered as a single oral dose or five days as feeding treated seeds, ranges from 1.44 mg/kg (in mallard duck) to 69 mg/kg [in brown-headed cowbird (*Molothrus ater*)] (FLECHTER u. PEDERSEN, 1988a,b). Nearly, all studies found out that diazinon is highly toxic by the acute oral route (FINK, 1976; HILL u. CAMARDESE, 1981; GRIMES u JABER, 1987). LC_{50} values for technical diazinon ranged from 3.9 mg/kg to 32 mg/kg (USPHS, 1995). Chronic reproductive effects in mallard ducks, such as significant reduction in the number of hatching survivors, were observed after the oral administration of diazinon throughout the breeding period (LOEC = 16.3 mg/kg) (MARSELAS, 1989).

Diazinon presents the following environmental characteristics: moderately persistent and mobile, is degraded by hydrolysis in water, by photolysis and microbial metabolism in soil and dissipates by volatilization on water or soil surfaces. The hydrolysis depends on the pH values and can take 12 to 138 days to be completed. It can be detected in soil layers for five to 20 days (HOWARD, 1991).
1.2.7 Carbaryl

Carbaryl belongs to the carbamate group and is one of the most widely used broad-spectrum insecticides in agriculture, professional turf management, ornamental production, residential pet (flea collars for dogs), lawn and garden markets (BAYER CROP SCIENCE®, 2012; USEPA, 2003a).

It is practically not toxic to birds such as ducks, quails, geese and pheasants (BRITISH CROP PROTECTION COUNCIL, 2000; USEPA, 2003a). Furthermore, it is highly toxic after an acute exposure in honey bees, estuarine or marine invertebrates, and other aquatic animals. Numerous studies with plasma of different bird species have shown that this carbamate reversibly inhibits ChE activity (ALIAS et al., 2011; OROPESA et al., 2013).

This carbamate can remain in soil for four to 72 days and is faster reduced in sandy, flooded and well aerated soils. It can be detected in plant leaves on average for three days. Carbaryl does not dissolve in water and because of this characteristic is commonly found in groundwater (VENAKATESWARLU et al., 1980; USEPA, 2003a).

1.2.8 Hydramethylnon

Hydramethylnon is an indoor and outdoor residential, industrial and agricultural amidinohydrazone insecticide for the control of imported fire and harvester ants, cockroaches, termites and pastures. It is a slow action poison and causes the death of the insects by disrupting the energy production in their cells (LOVELL, 1979).

Since the LD$_{50}$ falls in the range of 1828-2510 mg/kg, hydramethylnon is slightly toxic to practically non-toxic to avian species on an acute oral basis, depending on the bird species (US NATIONAL LIBRARY OF MEDICINE, 1995). Studies were conducted to establish the toxicity
of hydramethylnon in mallard duck and bobwhite quail, resulting in a slight toxicity on a subacute dietary basis in both cases (USEPA, 1998a). However, it has been suggested that hydramethylnon may cause chronic reproductive effects in avian species. Uncertainties regarding the potential adverse reproductive effects in birds could be reduced if avian reproduction toxicity data were available for the compound.

Hydramethylnon tightly binds to soil particles, so that its mobilization and availability in the environment is extremely low. It can persist in the ground for seven to 391 days (VOGUE et al., 1994). An important fact is that plants do not absorb this pesticide from the soil, so that any residues on the plant leaves are due to a direct contact during the application of the product (BACEY, 2000).

1.2.9 Fosetyl-Aluminum

Fosetyl-Aluminum is a systemic fungicide and bactericide, recommended for preventive applications on vegetables. It is practically not toxic to birds, aquatic organism and bees. Its acute toxicity in wild birds was measured by applying a single dose of it in bobtail quail (LD$_{50}$ > 8000 mg/kg) and Japanese quail (LD$_{50}$ = 4997 mg/kg) (FAO, 2008). In a short-term dietary toxicity test (duration five days) with bobwhite quails and mallard ducks (LD$_{50}$ > 20000 mg/kg) no effects were observed. In another subchronic dietary study in Japanese quails (> 1500 mg/kg for six weeks), no signs of toxicity were observed. It is degraded very fast in the soil to nontoxic components (USEPA, 2000).
1.2.10 Metalaxyl

Metalaxyl is a systemic fungicide used to control plant diseases caused by mycetes or water-mold fungi. It is used on many crops, residential and greenhouse crops such as ornamental plants, trees, shrubs and vines, lawns and turf.

Metalaxyl was practically not toxic in mallard ducks (LD$_{50}$ > 10000 mg/kg feed), and was slightly toxic in an acute toxicity study in mallard ducks (LD$_{50}$ = 1466 mg/kg). The risk to birds is minimal; however, studies on the impact of this fungicide on the avian reproduction are still needed (USEPA, 1998b; CFCAH-EU-Comission, 2010).

1.2.11 Ethoprophos

Ethoprophos is an insecticide-nematicide of the organophosphate group. It inhibits the activity of the cholinesterase and has been implicated in at least one bird kill, in which nine adult Canada geese (Branta canadensis) died in Georgia (HILL et al., 1975; HUDSON et al., 1979). It was also detected in the gastrointestinal tract of the geese and the brain cholinesterase activity was inhibited in the three birds tested (HUDSON et al., 1984). The avian oral LD$_{50}$ ranges from 4.21 to 61 mg/kg, while the avian dietary LC$_{50}$ ranges from 33 to 118 mg/kg in upland game birds and from 287 to 550 mg/kg in waterfowl (FINK et al., 1978; HUDSON et al., 1979).

Based on laboratory studies, the substance is fairly persistent (USEPA, 2006). It has a high solubility and can be moderately absorbed in soil. Because of this, ethoprophos contaminates water surface. In an aerobic soil metabolism study, a half-life of 100 days was reported (USEPA, 2006).
1.2.12 Oxamyl

Oxamyl is a non-persistent carbamate with systemic and contact insecticide-nematicide activities. It can cause cholinesterase inhibition in animals and humans, over-stimulating the nervous system and causing nausea, confusion and dizziness (HARTLEY u. KIDD, 1983).

Based on the effects in birds, it is considered highly toxic. The acute oral dose in bobwhite quails is 4.18 mg/kg (GRIMES u. JABER, 1988b). The oral LD$_{50}$ in male mallard ducks is reported to be 3.83 mg/kg and in female mallard ducks 2.61 mg/kg (DUDECK u. BRISTOL, 1981). A subacute exposure (i.e. over 28 days) to oxamyl at a dietary level 50 mg/kg, led to reproductive effects such as the reduction of egg production and egg fertility in mallard ducks (ROBERTS et al., 1982; HARTLEY u. KIDD, 1983).

The degradation of the active ingredient, depending on a number of chemical and microbial factors, can take between many days to several weeks. Oxamyl can be degraded very quickly in neutral and alkaline environments; it persists longer under acidic conditions. The photolysis of oxamyl seems to be activated in acidic water sources and not in soil (USEPA, 2007a). This carbamate has a half-life of up to four weeks under aerobic conditions and of less than seven days under anaerobic conditions. Field studies show that the applied oxamyl is absorbed from the superficial soil layer in less than seven days (USEPA, 2007a).

1.2.13 Chlorflurenol-Methyl

Chlorflurenol-methyl is an herbicide and plant growth regulator, mostly used as a post-emergent control of broadleaf weed. In bobwhite quails, no toxic effects were observed (oral LD$_{50} > 10$ mg/kg and dietary LD$_{50} > 5$ mg/kg) (ESTOP u. TESKE, 1969; PEDERSEN u. SOLATYCKI,
There is no data available on chronic exposure effects as well as reproduction studies in birds.

The persistence of chlorfluoreno-methyl in the environment is not exact. It seems to be highly mobile in soils and degrades very fast under field conditions (USEPA, 2007b).

1.2.14 Ethylene

The pesticide ethylene is an herbicide and a plant growth regulator. It belongs to the hydrocarbon chemical group and is a gas. Because of this, the exposure occurs via the lungs. Products containing this substance are considered to have a low toxicity and high volatility. Hence, exposure through treated foliage and foods as well as through the skin and lungs is minimal. No adverse effects were observed in ecological studies in fish and wildlife animals (USEPA, 1992).

1.3. The effect of organophosphates and carbamates on cholinesterase activity

Pesticides can affect the environment, humans and wildlife, including birds. MITRA et al. (2011) reported that organophosphates and carbamates do not accumulate in the food chain and are less environmentally enduring, but may have severe effects on birds. One of the most common causes of poisoning in birds is the intoxication with anti-ChE insecticides such as organophosphates and carbamates (HILL et al, 1995, FAIRBROTHER et al., 1996). According to RATTNER u. FAIRBROTHER (1991), birds show a higher sensitivity than mammals, which correlates with the particularly low activity of organophosphate and carbamate degrading enzymes.

The most widely used group of anti-ChE insecticides are the organophosphates, which include parathion methyl, chlorpyriphos, dimethoate, profenfos, diazinon and fenitrothion, the latter
primarily being used for grain storage and locust control (RADCLIFFE, 2002). Organophosphates and carbamates are anti-cholinesterase (anti-ChE) chemicals that inhibit esterases, including acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). AChE is an enzyme that hydrolyses the neurotransmitter acetylcholine (ACh) and thereby terminates cholinergic synaptic transmission (WALKER u. THOMPSON, 1991). AChE has a high specificity for ACh, which is affected by high pesticide concentrations. BChE is a less specific esterase with a higher affinity for butyrylcholine (BCh), a synthetic substrate, which is even inhibited at low pesticide concentrations (THOMPSON u. WALKER, 1994).

1.3.1 Butyrylcholinesterase

BChE is also known as plasma or pseudo-ChE. The enzyme is synthesized in hepatic cells (SVENSMARK, 1963; KUTTY, 1980), and its activity can be detected in plasma (MYERS, 1953). A low activity of the enzyme can be detected e.g. in the white substance of brain, liver, heart and fat tissue. According to several authors (STEDMAN et al. 1932, MENDEL u. RUDNEY, 1943; MYERS, 1953), BChE hydrolyzes benzoylcholine, butyrylcholine, propionylcholine and other choline related compounds. In contrast to AChE, BChE is not inhibited by increasing substrate concentrations (AUGUSTINSSON, 1949).

1.3.2 Acetylcholinesterase

According to the Enzyme Commission of the International Biochemistry Union (IBU), AChE is also called the specific ChE. The main function is to inactivate neurotransmission at the level of the synapses of the neurons. The biosynthesis of ACh occurs in nerve cells; ACh accumulates in the vesicles and is released after membrane depolarization. In this context, AChE catalyzes the
hydrolytic cleavage of ACh and stops the transmission of impulses within a few milliseconds. In order to do so, AChE binds to the anionic center of the esterase, which is acylated and releases a choline molecule. By splitting off the acetate group, the initial state of the enzyme is restored. The nerve cells take up acetate and choline, build up new ACh molecules and store them again inside vesicles (ZINKE, 2000).

AChE is detected in muscles, nervous system as well as in erythrocytes of mammals (ALLES u. HAWES, 1940; NACHMANSOHN u. ROTHENBERG, 1945). The enzyme is characterized by pronounced substrate specificity to acetyl-β-methylcholine, acetylthiocholine and ACh and is inhibited by increasing substrate concentrations (ALLES u. HAWES, 1940; NACHMANSOHN u. ROTHENBERG, 1945; AUGUSTINSSON, 1949).

Thiocholine esters such as acetylthiocholine, butyrylthiocholine and propionylthiocholine iodide can be used as substrates to perform the measurements. AChE has a high specificity and reacts only with acetylthiocholine iodide. BChE hydrolyzes all three substrates. In addition, there are species-specific substrate affinities in the case of both enzymes. MYERS (1953) and AUGUSTINSSON (1949) reported that AChE generally has a higher substrate affinity and, at low substrate concentrations, more effectively metabolized AChE than BChE.

Many studies in birds report the use of blood cholinesterase activity as biomarker of exposure to anti-cholinesterase agents such as organophosphates and carbamates. This activity has been widely used to assess the exposure and effects of these pesticides in populations inhabiting agricultural areas (WESTLAKE et al., 1981a, b; GARD u. HOOPER, 1993; SOLER-RODRIGUEZ et al., 1998; PARSONS et al., 2000; MAYACK u. MARTIN, 2003; RENDON-
VON-OSTEN et al., 2005; ROY et al., 2005; OROPESA et al., 2013). However, according to SINGH u. RIZVI (2013), the AChE in the erythrocyte membrane shows many properties similar to that the AChE in brain tissue and may thus be considered indicative of the central nervous cholinergic status.

1.3.3 Inhibition mechanism

The inhibition of the ChE by organophosphates is irreversible, whereby oximes are indeed able to slowly reactivate the inhibited ChE (WILSON et al., 1992). In a first step, the organophosphate leads to the formation of a reversible enzyme-inhibitor complex via a transphosphorylation reaction. Instead of acetylating the electronegative catalytic center of the esterase, an immediate phosphorylation by the electrophilic phosphorus central atom of the OP occurs. Once one of the ester groups bound to the phosphorus atom is eliminated, the organophosphate molecule becomes irreversibly bound to the enzyme and thereby prevents its catalytic activity (ALDRIGE, 1953).

1.3.4 Secondary effects in birds

HILL (2003) reported that the ecotoxicological effects of organophosphates and carbamates were less pronounced, but that they could slowly affect the animal populations. Vertebrates and invertebrates can be exposed to or even poisoned by pesticides in different ways by consuming seeds or plants treated with chemicals, by taking pesticides up in a granular form mistaking them for food, by ingesting dead or struggling poisoned insects and other animals or through inhalation, contaminated water and absorption through the skin (HILL, 1992; WILSON et al., 1992; FOSSI et al., 1996). The intoxication depends on factors such as type of the insecticide,
degree, duration and frequency of exposure, species variation and degree of environmental contamination (OSWEILER, 1996; WILSON et al., 1998; WILSON et al., 2005).

Worldwide, hundreds of incidents with organophosphate and carbamate-induced bird poisoning have been reported (MADISON, 1993; HOOPER 2002; FLEISCHLI et al., 2004). Both pesticide groups are responsible for 50-70% of acute poisoning cases, thereby affecting the enzyme acetylcholinesterase (AChE), whose activity serves to terminate synaptic transmission in neuromuscular junctions and cholinergic brain synapses (MITRA et al., 2011).

According to SMITH (1987), 50% of all organophosphates and 90% of all carbamates are extremely toxic to birds (lethal dose less than 40 mg/kg). If there is a concomitant exposure to several different pollutants, it could lead to an additive toxic effect. However, JOHNSTON u. BAYLIS (1995) reported cases, in which the intake of different toxins attenuated the toxic effect. The inhibition of AChE by such compounds leads to an increased concentration of ACh in the synapses, leading to the disruption of the normal functioning of the nervous system (WALKER u. THOMPSON, 1991). Acute toxicity can result in death by respiratory or cardiovascular arrest, or both, and sublethal exposures can lead to a range of biochemical, physiological and behavioral changes, since the cholinergic innervation of the body is nearly ubiquitous (FRYDAY et al., 1996; GRUE et al., 1997).

The toxicity forms can be classified as:

A) **Acute Toxicity**

The most possible route of exposure to organophosphates and carbamates is the consumption of seed or insects contaminated on their surface with lethal amounts of insecticides (PROSSER u. HARD, 2005).
B) **Sublethal Toxicity**

The ecology, physiology and behavior of the wild birds have been well studied and their alteration within the populations, because of human actions and pollution are of great public interest (BECKER, 2003). Sublethal effects of pesticides include, but are not limited to endocrine disruption, alterations in feeding behavior and a compromised immune system, all of which may affect avian reproduction. Therefore, pesticides can cause behavioral changes, loss of safe habitat and population decline up to local extinction of several bird species (MITRA et al., 2011). The following parameters can be affected by a sublethal intoxication with organophosphates and carbamates.

C) **Effects on feeding behavior**

Organophosphate and carbamate intoxication is often associated with anorexia and symptoms of gastrointestinal stress (GRUE et al., 1991). For example, long-term effects of very small amounts of organophosphates affect the feeding behavior of breeding red-winged blackbirds (*Agelaius phoeniceus*) (NICOLAUS u. LEE, 1999). Moreover, exposure to both groups of pesticides interferes with a bird’s ability to discriminate between contaminated and clean foods. Reduction in body weight following sublethal exposure with an average weight loss of 14% was also noted (MITRA et al., 2011). Such weight loss correlates with 55-77% AChE inhibition in European starlings (*Sturnus vulgaris*) after a single dose of dicrotophos (GRUE u. SHIPLEY, 1984). Lesions in the lateral hypothalamus due to pesticide exposure led to food avoidance and caused a sharp body weight reduction in birds (KUENZEL, 1994).
D) Effects on the endocrine system and reproductive behavior

Alterations in the reproductive behavior and gonadal development in birds (KUENZEL, 1994) have been noticed following acute sublethal exposure to organophosphates and carbamates due to hypothalamic lesions. Reduction in singing and displaying of throat feathers in the European starling (HART, 1993) and increased aggression in both sexes (GRUE et al., 1991) are strongly correlated with brain cholinesterase inhibition. In organophosphate-exposed mallards, their hatching success was reduced by 43% in comparison to controls because of an abnormal incubation behavior (nest abandonment and extended time away from the nests) (BENNETT et al., 1991). Alterations in the migratory behavior (VYAS et al., 1995), sexual behavior (GRUE u. SHIPLEY, 1981; HART, 1993), litter and clutch size (BENNETT et al., 1991) and parental care (GRUE, 1982) are due to reduced levels of reproductive hormones, which result from pesticide exposure.

It is possible that organophosphorus insecticides impair reproductive function by altering secretion of luteinizing hormone (LH) and progesterone (RATTNER et al., 1984). The decreased level of cholinesterase activity in testis and brain of adult male white-throated munia (Lonchura malabarica) is directly related to the increased number of degenerated germ cells after exposure to methyl parathion (MAITRA u. SARKAR, 1996). The exposure of adult male rose-ringed parakeets (Psittacula krameri) to methyl parathion resulted in impaired testicular function, which might be due to altered circulating levels of LH and testosterone in the circulating blood (MAITRA u. MITRA, 2008).
E) **Effect on thermoregulation**

Organophosphates and carbamates also affect the thermoregulation in birds. Acute sublethal exposure to organophosphates results in short-term hypothermia (GRUE et al., 1991). These pesticides induce a reduction in the body temperature of birds, which is often associated with a decrease in cholinesterase activity by more than 50% (CLEMENT, 1991). The correlation between low body temperature and pesticide toxicity appears to be the result of the impairment of thermoregulation, causing the inability of birds to withstand cold temperatures (MARTIN u. SOLOMON, 1991).

F) **Effect on the hematological system and immune system response**

Exposure to high doses of organophosphates can cause direct damage to cells and organs of the immune system and decrease immune functions. Histopathological changes in immune tissues and organs, cellular pathology, altered maturation, changes in lymphocytes and functional alterations in immunocompetent cells have been documented after organophosphate exposure (VOCCIA et al., 1999; AMBALI et al., 2010). Other effects include the direct damage of proteins and DNA (VIDEIRA et al., 2001). Organophosphates interfere with the immune response in animals through both anti-cholinergic and non-cholinergic pathways (BARNETT u. RODGERS, 1994; VIAL et al., 1996). Sublethal exposure of young chickens to chlorpyriphos and methidathion results in a reduction in the number of white blood cells, neutrophils and lymphocytes (OBAINEH u. MATTHEW, 2009).
1.4. Wild birds as biomonitors for environmental contamination

Monitoring of chemical concentrations in the environment is often performed by using certain animal species as surrogates (“biomonitors”). These are used to measure the concentration of pollutants needed to affect the organisms and ecosystems (FURNESS et al., 1993; LAM et al., 1999). The choice of biomonitoring species is very important. The species should be representative of the entire ecosystem and has to be receptive to contamination in order to be able to detect environmental pollution at an early stage (BURGER, 1993; FURNESS, 1993). Several pollutants representing a health risk for humans, such as dichloro-diphenyl-trichloroethane (DDT), have been shown to induce adverse effects in wild bird populations (RATCLIFFE, 1967).

Avian species have an unique place in the ecosystem. They constitute a diverse and evolutionary population and represent a large group in the tropical areas. The threats leading to their population decline are manifold and varied, but agriculture alone affects 87% of the globally threatened bird species (BLI, 2008). Healthy avian populations are indicators of ecological integrity, warning about environmental problems and ecosystem collapse. Avian populations have a central role in the ecosystem functioning and services, providing economic benefits like seed dispersal, pollination, recolonisation and restoration of disturbed ecosystems, as well as pest control (SEKERCIOGLU et al., 2004).

Birds have extensively been used in the past as biomonitors of environmental contamination with persistent organic pollutants (WALKER et al., 2001; HERZKE et al., 2003; LINDBERG et al., 2004). They are situated high in the food chain, thus accumulating high levels of organo-halogenated pollutants, and they are sensitive to environmental changes (FURNESS, 1993).
Biomarkers are intended to give information about the exposure to pollutants at an individual level. Biomarkers of exposure can be quite useful as an early warning signal before effects at more ecologically relevant levels (populations or communities) can be observed (GUIHERMINO, 2007).

However, it is important to mention that there are also some disadvantages when using birds as a biomonitoring species. Many birds are mobile and migrate over long distances, making it very difficult to relate contamination in the bird to a particular source. Moreover, some pollutants can be metabolized or excreted by the animal (LETCHER, 2000; VERRE AULT et al., 2005). Feces better reflect the excretion and metabolism of organic pollutants rather than their actual accumulation in the bird (DE VOS u. DE SCHRIJVER, 2005).

1.5. Pesticide contamination in feather and tissue samples

The use of hair, a keratinous tissue, has recently been evaluated as a method for the analysis of persistent organic pollutants (DAUBERSCHMIDT et al., 1998; COVACI et al., 2002; ALTSHUL et al., 2004; D’HAVE et al., 2005). Moreover, the analysis of hair to determine the concentrations of drugs (VILLAIN et al., 2004; BOUMBA et al., 2006) and contaminants (COVACI et al., 2001b; ALTSHUL et al., 2004; BOUMBA et al., 2006; D’ HAVE, 2006) has been successfully performed for several years. Since feathers are composed of a keratinous matrix as well, they are potentially useful to study the contamination with organic pollutants. In contrast to hair, which is continuously growing, feathers just grow for a certain period of time and are only connected to the blood stream (and its circulating pollutants) during this limited time period (JASPERS et al., 2004). Feathers have been used for monitoring heavy metal pollution for over 40 years (WEYERS, 1988; BURGER, 1993; JANSSENS et al., 2001).
While many biomonitoring studies on organic pollutants have previously focused on bird eggs, feathers have the advantage that they can be collected irrespective of season, age or sex. Bird feathers have previously been used for monitoring heavy metals in numerous studies, but the use of feathers as monitors of persistent organic pollutants (POPs) (such as polychlorinated biphenyls [PCBs], DDT and organochlorine pesticides) have only recently been investigated (DAUWE et al., 2005; JASPERS et al., 2006b; VAN DEN STEEN et al., 2007).

1.6. Wild bird species

All four bird species included in this study are small non-migratory birds, living in the forests close to pineapple cultivation areas. The variable seedeater, *Sporophila americana*, has an approximate size of 10.5 cm and an average weight of 11 g. The males have a black color and a very typical convex pinnacle, while the females are coffee brown-colored. *S. americana* lives frequently in groups with other species like *Volatinia jacarina* because of the similar food preferences, including grass, tree and shrubbery seeds, berries and some insects as a protein source. The males sing in the high parts of the trees. The reproduction period is between May and August. The blue-black grassquit, *Volatinia jacarina*, exhibits an average size of 10 cm and a weight of 9.5 g and has a conical black bill. The male is glossy blue-black with a black tail and wings. The female has dull brown-colored upperparts and dark-streaked buff underparts. The areas of habitat include grass, herbs, bushes and shrub fields as well as the border of forest areas. Generally, they have similar feeding habits like *S. americana*. Their reproduction period is between June and October. The trick-billed seed-finch, *Sporophila funerea*, is 11.5 cm in size and weighs 13.5 g. The bill is more robust and conical than that of *S. americana*. The adult male is
colored in a deep black color, except for the lining of the wings and the bases of the primary feathers, which are white. The bill is black and the legs are dark grey. The female is deep dark brown on its upper part and the wings and the tail show blackish brown edges. The ventral side of the animal is between dark and opaque at the throat, while breast and flanks are dark coffee brown and the lining of the wings is white. It lives in grass, shrubby and bushy areas, and on fields with tall grass and swamps. In contrast to *S. americana*, this bird species only lives in pairs and not in big groups on trees. It feeds on grass seeds, small berries and insects. The period of reproduction is from April to September. Finally, the scarlet-rumped tanager, *Ramphocelus passerinii*, is 16 cm big and weighs 31 g. It has a thick bill. The male is of a velvet black colour, except for the lower back, which is colored in an intense orange. The female shows a brownish grey head and an ochraceous olive upperparts, with a paler and shiny rump. The wings and tail are dusky and the throat is greyish. The rest of the lower part is ochraceous olive, brighter on the chest. The peak is often dully grey. The scarlet-rumped tanager ingests a lot of fruits as well as some berries and preys such as insects and spiders in the foliage. The species lives in secondary forests, scrubs and forest borders. It is reproductively active between March and August (STILES u. SKUTCH, 2007).

1.7. Pesticide analysis with the QuEChERS method

The quick, easy, cheap, effective, rugged and safe method (known as QuEChERS) was developed by ANASTASSIADES u. LEHOTAY (2003) and it is a standardized protocol for multiresidue pesticide analysis in fruits, vegetables and other food matrices, recognized by the EU since 2007. This method consists of two steps: liquid-liquid sample extraction and purification by solid phase extraction (SPE). Follow-up studies have further validated this technique for more than 200
pesticides, improved the analytical precision for the remaining few problematic analytes and tested it in fat-containing matrices (LEHOTAY et al., 2005a). The method uses a single-step buffered acetonitrile (MeCN) extraction, while anhydrous magnesium sulfate (MgSO$_4$) removes water from the sample and induces the liquid-liquid partitioning. For the cleanup step, a simple, inexpensive, and rapid technique called dispersive solid-phase extraction (SPE) is conducted using a combination of primary secondary amine (PSA) sorbents to remove fatty acids (among other components) and anhydrous MgSO$_4$ to reduce the remaining water in the extract. Then, the extracts are concurrently analyzed by liquid and gas chromatography (LC and GC) combined with mass spectrometry (MS) to determine a wide range of pesticide residues. In 2005, acetate salts were added in order to buffer the liquid–liquid extraction and avoid the degradation of base-sensitive pesticides. This method ensures the successful extraction of highly polar and highly acidic and basic pesticides (LEHOTAY et al., 2005b). Therefore, SPE has gained popularity as a tool for the isolation, concentration and purification of analytes from complex matrices (LEHOTAY et al., 2005c). In addition, SPE involves a simple analytical procedure that produces clean extracts and leads to high recovery rates. The non-polar octadecyl (C18) bonded silica is the widely used sorbent in this method (KUMAZAWA u. SUZUKI, 2000).
2. Objectives

2.1. General objective

The primary aim of this study was to characterize the AChE and BChE activities in four wild bird non-migratory species (S. americana, S. funerea, V. jacarina and R. passerinni), captured at the forest-crop interface of conventional and organic pineapple plantations.

2.2. Specific objectives

- To standardize assay conditions to measure AChE and BChE activity in brain tissue and serum, respectively.
- To compare the AChE and BChE activity of the different species (male and female animals) in conventional and organic crops.
- To analyze extracts of feathers, feces, skin and soil by LC-q-TOF-MS and determine the accumulation level of pesticides used in the pineapple cultivation areas (organic and conventional).
- To explore the feasibility of using any of these species as a toxicity biomonitor in pineapple plantations.
3. Materials and methods

3.1. Enzyme activity measurements

3.1.1 Sample collection

Materials:

<table>
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<tr>
<th>Product</th>
<th>Type /Catalog Nr.</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ketamid® (ketamine + midazolam)</td>
<td>50 ml</td>
<td>Holliday-Scott®</td>
<td>Argentina</td>
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<td>Microcentrifuge tube</td>
<td>1.5 ml</td>
<td>Eppendorf®</td>
<td>Germany</td>
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Equipment:

<table>
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<th>Company</th>
<th>Country</th>
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<td>5415C</td>
<td>Eppendorf®</td>
<td>Germany</td>
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<td>MVE</td>
<td>SC Series11/7</td>
<td>Millenium</td>
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<tr>
<td>CX</td>
<td>100</td>
<td>Taylor Wharton</td>
<td>USA</td>
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This study included 196 blood and 197 brain samples of the four species described in the Introduction section and living in the gallery forests surrounded by pineapple-growing areas of one Costa Rican pineapple plantation in the Northern Region, also called Huetar Norte Region. The exact geographical coordinates of the conventional and organic points were identified and saved using a navigation system (Figure 1). The samples were collected in the period between February and April 2012 using mist nets in the morning hours, between 5:30 am and 9:30 am, in order to minimize stress and possible diurnal variation in enzyme activities (GARCIA-
RODRIGUEZ et al., 1987; THOMPSON et al., 1988; COBOS et al., 2010). The captures were done in the border between gallery forests and pineapple areas and close to water sources such as lakes, ponds, streams and rivers (Figure 2). Most of those water sources are used for irrigation of the pineapple regions or serve as drainage. None of the birds, which were observed during the field work, presented symptoms of illness or weakness.

After the capture, each bird was put in a separate cloth bag and immediately put to sleep with an overdose of a combination of ketamine (50 mg/ml) and midazolam (2 mg/ml; Holliday-Scott®, Argentina) applied parenterally, the recommended dose for birds being 0.04 ml/100 g body weight for stress minimization without reducing the blood supply (WHELER, 1993). Thereafter, the birds were decapitated for the collection of the blood samples in microcentrifuge tubes (Eppendorf®, Germany). Plasma was separated from the erythrocytes by centrifugation (Eppendorf®, Germany) at 7000 rpm for 5 minutes, and the brain of each bird was excised for the measurement of AChE activity. During the sample collection period of time and the transport to the laboratory, all plasma and brain samples were kept in liquid nitrogen (Millenium & Taylor Wharton, USA). Then, samples were stored at -80°C until they were analyzed.
Figure 1. A) Regional map of Costa Rica indicating the collecting area (black square). B) Map of the pineapple plantations in Northern Costa Rica (green zones indicate the organic lots and the yellow ones the conventional lots; white points mark organic sample points and black points mark conventional ones). Source: Soil and More International, Dole Food Company (2011).
Figure 2. Sample point in a conventional pineapple-growing area: Mist nets were installed at the border between the pineapple plantation areas and the surrounding gallery forests with water sources.

3.1.2 Plasma cholinesterase activity measurements

Kits:

<table>
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<th>Type / Catalog Nr.</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial kit</td>
<td>serum cholinesterase</td>
<td>Bio-Tec® International S.A.</td>
<td>Costa Rica</td>
</tr>
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Equipment:

<table>
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<td>Scientific Accumet</td>
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<td>Fisher Scientific®</td>
<td>USA</td>
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<tr>
<td>LAMBDAA 35</td>
<td>UV/Vis Systems</td>
<td>Perkin Elmer®</td>
<td>USA</td>
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</table>

ChE activity was measured by using a commercial kit (Bio-Tec® International S.A., Costa Rica) based on a method described by ELLMAN et al. (1961), modified by HILL u. FLEMING (1982) and recommended by the manufacturer. It uses propionyl thiocholine iodide as a specific substrate and dithiobisnitrobenzoate as color reagent. The change of color is proportional to the enzyme activity. All assays were measured at 0, 15, 30 and 45 seconds to obtain the average values in the spectrometer (model J35, Perkin Elmer®, USA) at 30°C and a wavelength of 450 nm.

3.1.3 Acetylcholinesterase activity measurements in brain tissue

Kits:

<table>
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<th>Product</th>
<th>Type / Catalog Nr.</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
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<tr>
<td>Commercial kit</td>
<td>erythrocyte</td>
<td>Bio-Tec®</td>
<td>Costa Rica</td>
</tr>
<tr>
<td>DC Protein Assay</td>
<td>cholinesterase</td>
<td>International S.A.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bio-Rad-500-0112</td>
<td>Bio-Rad®</td>
<td>USA</td>
</tr>
</tbody>
</table>
The complete brain tissue sample (frontal cortex and basal ganglia) was homogenized with a sterile 1ml syringe on an ice bath. Then, 0.01 g of the homogenized sample were transferred to a new tube and 0.05 M Tris-base buffer (pH 8.0; Sigma-Aldrich® USA) were added at a ratio of 1:9, mixed and homogenized on ice again, until the tissue was completely disintegrated.
**Quantification of proteins**

The protein content in all the samples was determined according to the method of BRADFORD (1976) adapted to a microplate readout. A commercial kit was used for this quantification of the proteins (DC\textsuperscript{TM} Protein Assay 500-0112 BioRad\textsuperscript{®}, USA), and the measurements were performed by using an ELISA reader (Shimadzu\textsuperscript{®}, Japan). Different dilutions (0.2, 0.4825, 0.765, 1.0475 and 1.33 mg/ml) of a protein standard were prepared using the same buffer. Subsequently, a 1:20 dilution of each sample was performed in triplicate in an ELISA 96 well plate. The methodology recommended by the manufacturer was used. The samples were left at room temperature for 15 minutes and then analyzed in the ELISA reader with a 650-750 nm filter. Finally, an X/Y scatter chart of standards was developed and the R\textsuperscript{2} value with its respective equation was determined.

**Determination of acetylcholinesterase activity in brain tissue**

For the determination of ChE activity, the same procedure described for the measurement of erythrocyte ChE (Biotech International\textsuperscript{®}, Costa Rica) was used, but in this case, it included another substrate (acetylthiocholine iodide), and the absorbance was measured only at 0 and 30 seconds at 450 nm according to the instructions of the manufacturer.

AChE activity was determined in brain homogenate supernatants and expressed in Units (U) per grams (g) of protein (one U equals to one nmol acetylcholine hydrolyzed per minute). The supernatant protein concentration of the brain homogenates was adapted to the microplate and then determined according to LOWRY et al. (1951) with BAS as standard. The methodology recommended by the manufacturer was used.
3.1.4 In vitro inhibition of butyrylcholinesterase by carbaryl

Kits and materials:

<table>
<thead>
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<th>Type / Catalog Nr.</th>
<th>Company</th>
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<td>Germany</td>
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<td>Ethanol</td>
<td>459844</td>
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<td>ab138871</td>
<td>Abcam®</td>
<td>United Kingdom</td>
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<tr>
<td>Commercial kit</td>
<td>serum cholinesterase</td>
<td>Bio-Tec® International S.A.</td>
<td>Costa Rica</td>
</tr>
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</table>

As a commercial kit control and to evaluate the inhibitory potential of carbaryl on wild bird BChE, an in vitro test was performed according to OROPESA et al. (2013) with some slight modifications. For this control experiment, two blood samples of unexposed African grey parrots (Psittacus erithacus) from the Clinic for Pets, Reptiles, and Feral Birds of the University of Veterinary Medicine Hannover were collected. These two samples were centrifuged. The obtained plasma was pooled in order to minimize specific individual differences and to increase the available amount.

Carbaryl with a purity of 98% (Sigma Aldrich®, Germany) was diluted in ethanol at 0.781, 3.125 and 12.5 and 50 µg/ml. The effect of carbaryl on the ChE activity was determined after an incubation period of 30 minutes at 25°C in darkness. Subsequently, 5 µl of each stock solution
was added to 495 µl of a pooled sample, while 5 µl of ultrapure water was used for the blank assay samples. Additional controls were incubated with 5 µl ethanol under the same conditions. The commercial kit was compared with another one (Abcam®, United Kingdom) and the BChE inhibition was calculated in percentage. The methodology recommended by each manufacturer was used in both kits.

3.2. Sample collection for pesticide contamination and extraction method

Materials:

<table>
<thead>
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<th>Product</th>
<th>Type / Catalog Nr.</th>
<th>Company</th>
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<td>271004-11</td>
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<tr>
<td>Sodium chloride</td>
<td>S3014</td>
<td>Sigma Aldrich®</td>
<td>Germany</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>791741-500g</td>
<td>Sigma Aldrich®</td>
<td>Germany</td>
</tr>
<tr>
<td>Isolute MSPD C18</td>
<td>9370-0100</td>
<td>Argonaut Technologies</td>
<td>Hungary</td>
</tr>
</tbody>
</table>

Equipment:

<table>
<thead>
<tr>
<th>Product</th>
<th>Type / Catalog Nr.</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vortex</td>
<td>Genie 2</td>
<td>VRW International©</td>
<td>Canada</td>
</tr>
<tr>
<td>Ultrasonic bath</td>
<td>Branson 3800</td>
<td>Emerson Industrial Automation©</td>
<td>USA</td>
</tr>
<tr>
<td>Analytical balance</td>
<td>PC 180</td>
<td>Mettler Toledo</td>
<td>Switzerland</td>
</tr>
<tr>
<td>Lyophilisator</td>
<td>Sentry 2.0</td>
<td>Virtis SP Scientific®</td>
<td>USA</td>
</tr>
</tbody>
</table>
**Feathers**

A total of 201 feather samples (109 from conventional fields and 92 from the organic fields) from all the four bird species described in the Introduction section were collected directly after euthanasia and put in a small plastic bag. Sample feathers from the upper part, wings and tail were thereby mixed. All samples were kept at 0°C in the field, at -10°C during transport and stored at -20°C in the laboratory until they were further processed.

The use of powerful ultrasound significantly improves the extraction of organic compounds contained within the body of plants and seeds. The ultrasound treatment allows a greater penetration of the solvent into the materials and improves mass transfer (MASON et al., 1996). Most of the compounds were extracted during the first ten minutes of sonication (MASON u. ZHAO, 1994). Depending on the wave intensity, exposure time, membrane characteristics and medium type, ultrasonic waves can induce mechanical, thermal and biochemical effects in the frame of a large range of applications in the food industry (ZENKER, 1998). The effects of an ultrasonic bath are to facilitate the extraction process and to reduce the extraction time compared to other methods. The ultrasonic extraction has successfully been applied for the determination of environmental pollutants and pharmacologically active substances (MARTINEZ, 2004).

In the present study, an ultrasonic bath (Emerson Industrial Automation©, USA) was used to optimize the extraction of pesticides in the feather samples. All samples were processed the same day, cut in small pieces (0.1-0.5 mm), weighed and put in a clean conic tube. Thereafter, 5 ml acetonitrile (Sigma Aldrich®, Germany) were added and the tubes were immersed in an ultrasonic bath at a 30°C for 15 minutes and with 40 kHz frequency.
In the case of the feathers, all samples were mixed with 5ml acetonitrile and 1% acetic acid before the ultrasonic extraction, and after a centrifugation 2 ml of extract were gathered.

**Skin and feces**

All 201 skin samples (109 from the conventional pineapple-growing areas and 92 from the organic ones) were collected during the field work. Skin tissue from the breast area was taken and then carefully placed in individual Eppendorf® tubes. Feces samples (109 from the conventional pineapple cultivation zones and 85 from the organic ones) were taken very carefully directly from the rectal area and distal part of the colon after the necropsy on the field. Every test tube were immediately put in liquid nitrogen tanks and maintained in them during the field work and the transport period. In the laboratory, they were kept at -80°C until the following preparation process was performed.

The skin samples were mixed regarding species, day and place of collection. The same procedure was applied for the feces samples. They were weighed and inserted into a lyophilizer for 24 hours (Virtis SP Scientific®, USA) to facilitate the consequent preparation and maceration in order to ensure the extraction of the water in the sample.

The pools of skin and feces samples were processed independently of the final volume using the QuEChERS-method by mixing them with 2 ml acetonitrile containing 1% acetic acid (Sigma Aldrich®, Germany) and then using a Vortex mixer (VRW International®, Canada) for one minute. A salt mixture (0.4 g magnesium sulfate; 0.1 g sodium chloride and 0.05 g sodium acetate; Sigma Aldrich®, Germany) was added, mixed manually and centrifuged for seven minutes at 10°C and 4500 rpm. The resulting supernatant was left for 1 hour at -20°C in order to allow for the separation of the fat in the sample. It was then centrifuged, the extraction tube was
changed and 50 mg of C18 sorbent (Argonaut Technologies, Hungary) were added. The mixture was vortexed, centrifuged again and 1ml aliquot was taken.

**Soil**

Soil samples were taken from all the places of collection (four in the conventional and four in the organic pineapple cultivation areas), put in plastic bags and kept at 0°C during the field work and at -10°C during the transport. In the laboratory, they were kept at -20°C until the following preparation process step was performed. Soil samples were taken from each collection point and very well mixed. Then, five grams were weighed for each extraction. The soil samples of each conventional point were compared with four soil samples from the organic zones.

Because of the greater weight of the soil samples, higher amounts of the reagents were used: 5 ml acetonitrile with 1% acetic acid, a salt mixture consisting of 2 g sulfate, 0.5 g sodium chloride and 0.5 g sodium acetate and for the clean-up step 0.5 g C18.

### 3.3. Pesticide Analysis by UHPLC-TOF-MC

**Reagents:**

<table>
<thead>
<tr>
<th>Product</th>
<th>Type / Catalog Nr.</th>
<th>Company</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexakis</td>
<td>AS441921</td>
<td>Apollo Scientific Limited®</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>56467-2ml</td>
<td>Sigma Aldrich®</td>
<td>Germany</td>
</tr>
<tr>
<td>Methanol</td>
<td>MS Quality</td>
<td>Carl Roth®</td>
<td>Germany</td>
</tr>
<tr>
<td>Ammonium formate</td>
<td>70221-25g</td>
<td>Sigma Aldrich®</td>
<td>Germany</td>
</tr>
<tr>
<td>Sodium formate</td>
<td>247596-100g</td>
<td>Sigma Aldrich®</td>
<td>Germany</td>
</tr>
</tbody>
</table>
An aliquot of 1 ml of each sample was sent to the Max-Rubner-Institute in Detmold, Germany, to be processed and analyzed in an electrospray interface time-of-flight mass spectrometer system (ESI-q-TOF-MS) (Brucker Corporation®, Germany) for qualitative and quantitative Multi-Target Pesticide screening.

- **Chromatography**

Chromatographic separations of the extracts were performed with a UHPLC (Dionex™, Germany) consisting of a solvent rack, an autosampler, a binary pump and an UV detector equipped with a Van Guard Precolumn (Waters®, USA) and a reversed-phase C18 analytical column of 2.1 mm x 100 mm and 2.2 µm particle size (Dionex™, Germany). The separation was
performed at a temperature of 30° C in gradient mode using the mobile phases A = H_2O/methanol (90/10) and B = methanol with 5mM ammonium formate in 0.01% HCOOH.

The following table shows the chromatographic conditions:

Table 2. Chromatographic conditions regarding the used percentage of the mobile phases A and B, the flows and the retention times.

<table>
<thead>
<tr>
<th>Retention Time [min]</th>
<th>Flow [ml/min]</th>
<th>%A</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>0.1</td>
<td>0.2</td>
<td>99</td>
<td>1</td>
</tr>
<tr>
<td>1.0</td>
<td>0.2</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>14</td>
<td>0.4</td>
<td>99.9</td>
<td>0.1</td>
</tr>
<tr>
<td>16.0</td>
<td>0.48</td>
<td>99.9</td>
<td>0.1</td>
</tr>
<tr>
<td>16.1</td>
<td>0.48</td>
<td>1.0</td>
<td>99.0</td>
</tr>
<tr>
<td>19</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.1</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.2</td>
<td>1.0</td>
<td>99.0</td>
</tr>
</tbody>
</table>

Injection volume: 1 µl

The software control was conducted by using the programs HyStar™ and OTOF-control™ (Brucker Corporation©, Germany). The interpretation of the obtained results was supported by the database (called BDAL-Pestide-DB_POS_RT), and finally the data was analyzed by Target Analysis 4.1 and Data Analysis 1.3 software (Brucker Corporation©, Germany).

- **LC-Electrospray time-of-flight spectrometry**

All the extracts were analyzed for polar pesticides using an UHPLC, connected to a qualitative ESI-q-TOF-MS operating in positive ion mode using the following parameters:
Table 3. Parameters used in the UHPLC and flight mass spectrometer.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Source</td>
<td>ESI positive mode</td>
</tr>
<tr>
<td>Calibrant</td>
<td>1 mM sodium formate/acetate in iPrOH/H₂O (50/50) with 0.2% acid</td>
</tr>
<tr>
<td>Calibrant for Lock Mass Calibration</td>
<td>Hexakis(1H,1H,2H-perfluoroethoxy) phosphazene 0.1 mg/mL (iPrOH) at m/z 621.19.</td>
</tr>
<tr>
<td>Mass range</td>
<td>50-1000 m/z</td>
</tr>
<tr>
<td>Spectra Rate</td>
<td>1 Hz</td>
</tr>
<tr>
<td>End Plate Offset</td>
<td>500V</td>
</tr>
<tr>
<td>Capillary</td>
<td>2500 V</td>
</tr>
<tr>
<td>Nebulizer</td>
<td>2.5 Bar</td>
</tr>
<tr>
<td>Dry Gas</td>
<td>8.0 l/min</td>
</tr>
<tr>
<td>Dry temperature</td>
<td>200°C</td>
</tr>
<tr>
<td>Funnel 1 RF</td>
<td>200.0 Vpp</td>
</tr>
<tr>
<td>Quadrupole Ion Energy</td>
<td>4.0 eV</td>
</tr>
<tr>
<td>Collision Cell/Collision Energy</td>
<td>4.0 eV</td>
</tr>
<tr>
<td>Funnel 2 RF</td>
<td>200 Vpp</td>
</tr>
<tr>
<td>Hexapole RF</td>
<td>50 Vpp</td>
</tr>
<tr>
<td>Low mass</td>
<td>50.00 m/z</td>
</tr>
<tr>
<td>Pre Pulse Storage</td>
<td>6 µs</td>
</tr>
</tbody>
</table>

A total volume of 50 - 100 µl TPP (100 µg/ml) was added to all the samples as an internal control.

Before each individual sample extract measurement, the MS system was calibrated by using a solution of sodium formate/acetate (Sigma Aldrich®, Germany) in isopropanol / 0.2% formic acid (1:1, v/v). The mass calibration for the pesticide measurement was done by using the internal reference mass of the Hexakis solution (Apollo Scientific Limited®, United Kingdom).
• **Calibration and validation parameters of the standards**

For the linearity measurements, an internal standard of each pesticide at a concentration of 10 µg/ml as well as dilutions with different concentrations ranging from 0.01 µg/ml to 2 µg/ml and from 0.5 µg/ml to 10 µg/ml were prepared. The different extracts and the solvent-based calibration standards were measured in the full scan mode based on accurate mass and isotope pattern, mass accuracy, resolution, limits of detection and quantification. Linearity, dynamic range and peak area reproducibility were evaluated for the pesticide mixture using Data Analysis 4.1 and Target Analysis 1.3 software.

The limit of detection (LOD) was based on the mass giving a signal equivalent to three times that of the noise (S/N = 3). The limit of quantification (LOQ) was set based on the mass giving a signal equivalent to ten times that of the noise (S/N = 10). A LOD of 0.01 µg/mL and a LOQ of 0.1 µg/mL were determined.

**Table 4. Calibration curves according to the analyzed pesticides.**

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Correlation coefficient r (low calibration curve)</th>
<th>Correlation coefficient r (high calibration curve)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diuron</td>
<td>0.9997</td>
<td>0.9937</td>
</tr>
<tr>
<td>Ametryn</td>
<td>0.9996</td>
<td>…</td>
</tr>
<tr>
<td>Bromacil</td>
<td>0.9994</td>
<td>0.9935</td>
</tr>
</tbody>
</table>

The calibration curves were used to quantify samples that contained one or more pesticides. The quality check consisted of the evaluation of the calibration curves and regular analyses of procedural blanks, solvent blanks and duplicate sample analyses (Table 4). Each compound was characterized by its own retention time and the masses of the two conducted measurements. Each
sample was injected twice and the instrumental performance was checked according to the correct signal area of TPP and its retention time.

- **Quantification**

Each sample was injected twice in order to prove that the system measurements were correct and that the internal standard TPP appeared at the same retention time. For the automatic evaluation of the collected data, the software Target Analysis 1.3 was used, which is connected to a database. The retention time took 30 seconds.

Finally, the information obtained from the samples was processed with the software Data Analysis 4.1, with which it was tested whether the signals of the detected individual pesticides were below the LOD or within the noise area. A manual integration of the individual peaks was also performed. Whether, the concentration of the pesticides in the samples fell within the linear concentration range was also analyzed.

3.4. **Statistical model**

Results were analyzed by the software program GraphPad Prism 6 (GraphPad Software©, USA) to obtain descriptive statistics of the data. The mean values and standard error of mean (SEM) were expressed in all possible cases. After checking sample distribution and variance homogeneity, all non-parametric values were tested by performing the Kruskal-Wallis test and the Mann-Whitney test.
4. Results

4.1. Environmental observations

In the conventional pineapple cultivation areas, it was possible to observe fumigation trucks and fumigation schedules written on boards at the entrance of each zone (Figure 3). The boards pointed out date, hour, area of application and commercial pesticide names (Figure 4). The use of chlorpyriphos, copper sulphate, calcium hydroxide and products with organic compounds (D-limonene) and extracts of plants such as pepper (*Capsicum annum*), garlic (*Allium sativum*), onion (*Allium cepa*) and *Neurolaena lobata* were reported on those boards.

Figure 3. Application of agrochemicals in a conventional pineapple area.
Figure 4. Agrochemical application board in a conventional pineapple area.

In the organic areas, the pineapples were only covered with black plastic bags to protect them against plagues (Figure 5). Information on the possible presence of organic pesticides in these bags was not available.
Figure 5. View of an organic pineapple area.

During the capture of the birds, they were observed in groups around the pineapple areas (looking for shelter or seeds in the gallery forests) or within them, especially early after the sunrise. In those groups there were birds of the same species as well as birds of different species flying together (Figure 6).
4.2. Enzyme activity

4.2.1 Plasma butyrylcholinesterase activity

Descriptive statistics of plasma BChE activity (IU/L) measured in free-living wild birds by species and pineapple-growing area were performed (Table 5). The analyses of the minimum-maximum mean ranges in these four birds species in the conventional and the organic areas indicated a large range of BChE activities, whereby a reduced activity was observed in the conventional bird groups, even though the total number of animals from these areas was higher than that of birds from the organic areas.
The BChE activity values (maximum, average and minimum ranges) of birds sampled in the organic areas (n = 88) were higher when compared to those of the conventional ones (n = 108), as shown in Figure 7. This result was also analyzed per bird species: the birds from the conventional areas in three out of four studied species showed lower levels of plasmatic BChE, except for *R. passerinii*, whose maximum BChE mean ranges were higher in the conventional zones, even though the number of sampled *R. passerinii* was the same in both areas (n = 17). It is also remarkable that *S. americana* showed the highest maximum, mean and minimum levels of BChE activity in both areas (Figure 8).

<table>
<thead>
<tr>
<th>Descriptive statistic values</th>
<th>Sporophila americana</th>
<th>Sporophila funerea</th>
<th>Volatinia jacarina</th>
<th>Ramphocelus passerinii</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional n=46</td>
<td>Organic n=51</td>
<td>Conventional n=23</td>
<td>Organic n=5</td>
</tr>
<tr>
<td>Mean</td>
<td>12698</td>
<td>13625</td>
<td>5014</td>
<td>7840</td>
</tr>
<tr>
<td>SD</td>
<td>4550</td>
<td>4694</td>
<td>2285</td>
<td>2816</td>
</tr>
<tr>
<td>Minimum</td>
<td>4900</td>
<td>5500</td>
<td>2633</td>
<td>5267</td>
</tr>
<tr>
<td>Maximum</td>
<td>22300</td>
<td>32700</td>
<td>11067</td>
<td>10967</td>
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<tr>
<td>Lower 95% CI of mean</td>
<td>11347</td>
<td>12304</td>
<td>4026</td>
<td>4343</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>14049</td>
<td>14945</td>
<td>6003</td>
<td>11337</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Descriptive statistic values</th>
<th>Total in conventional areas n=108</th>
<th>Total in organic areas n=88</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>8926</td>
<td>11059</td>
</tr>
<tr>
<td>SD</td>
<td>4854</td>
<td>5051</td>
</tr>
<tr>
<td>Minimum</td>
<td>2633</td>
<td>3600</td>
</tr>
<tr>
<td>Maximum</td>
<td>22300</td>
<td>32700</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>8000</td>
<td>9989</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>9852</td>
<td>12310</td>
</tr>
</tbody>
</table>

Finally, the gender was included as the last variable (Figure 9). No normal distribution was observed by the statistical model in any of the four wild bird species. The Kruskal–Wallis test
was applied in the case of *Sporophila americana*, *Volatinia jacarina* and *Ramphocelus passerinii* for comparison of males vs. females and organic areas vs. conventional areas. An exception was made for *Sporophila funerea* because of the small amount of samples per gender collected in the organic area (total number of samples = 5). Generally, the female and male representatives of *S. americana* in the organic pineapple areas had higher BChE activities that those in the conventional ones. In *V. jacarina* and *R. passerinii* all values of the males appeared to be lower than those of the females, but without reaching any statistically significance. The *S. funerea* males from the conventional and organic areas showed similar BChE activity values.

![Figure 7. Butyrylcholinesterase (BChE) activity of all samples by pineapple cultivation area.](image)
Sporophila americana

Volatinia jacarina

BChE (IU/L)

Conventional  Organic

Conventional  Organic
Figure 8. Butyrylcholinesterase (BChE) activity per species in each growing area.
Volatinia jacarina (Kruskal-Wallis Test)

Sporophila americana (Kruskal-Wallis Test)
Figure 9. Butyrylcholinesterase (BChE) activity in serum by gender, sampled species and the pineapple-growing areas.
4.2.2 Acetylcholinesterase activity in brain tissue

Descriptive statistics of brain AChE activity (IU/g tissue) measured in free-living wild birds by species and pineapple-growing area were also evaluated (Table 6). In this case, the analysis of all the minimum-maximum mean ranges showed differences in the AChE activities between growing areas (Figure 10) and bird species. The largest range and the highest enzyme activity were observed in *V. jacarina* living within the organic zones. Interestingly, it was noted that *R. passerinii* in the organic areas reported the lowest AChE brain activity values, even though its mean value was somewhat higher than the mean value of their representatives in the conventional areas (Figure 11).

The results of AChE activity measurements in the brain tissue were also compared according to gender, species and growing area (Figure 12). *S. americana* of the conventional area showed a significant difference between males and females (p = 0.0356). In the case of *V. jacarina* and *R. passerinii*, the females showed in general higher enzyme activities than males, while the opposite was observed for *S. funerea*. The *R. passerinii* and *S. funerea* males of the organic pineapple cultivation areas showed higher values of AChE activity than the males of the conventional ones. No differences were found between the AChE values of males and females of *V. jacarina* in the conventional area. The highest level of AChE activity was in general found in *V. jacarina* females captured in the organic areas.
### Table 6. Descriptive statistics of brain AChE activity (IU/g tissue) measured in free-living wild birds in the conventional and organic pineapple-growing areas

<table>
<thead>
<tr>
<th>Descriptive statistic values</th>
<th><em>Sporophila americana</em></th>
<th><em>Sporophila funerea</em></th>
<th><em>Volatinia jacarina</em></th>
<th><em>Ramphocelus passerinii</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conventional n=46</td>
<td>Organic n=54</td>
<td>Conventional n=22</td>
<td>Organic n=5</td>
</tr>
<tr>
<td>Mean</td>
<td>4548</td>
<td>4350</td>
<td>4462</td>
<td>4497</td>
</tr>
<tr>
<td>SD</td>
<td>1599</td>
<td>1378</td>
<td>1304</td>
<td>2263</td>
</tr>
<tr>
<td>Minimum</td>
<td>1946</td>
<td>2270</td>
<td>1946</td>
<td>2811</td>
</tr>
<tr>
<td>Maximum</td>
<td>8973</td>
<td>8216</td>
<td>7135</td>
<td>8216</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>4073</td>
<td>3974</td>
<td>3884</td>
<td>1687</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>5023</td>
<td>4726</td>
<td>5040</td>
<td>7307</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Descriptive statistic values</th>
<th>Total in conventional areas n=106</th>
<th>Total in organic areas n=91</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>4453</td>
<td>4375</td>
</tr>
<tr>
<td>SD</td>
<td>1438</td>
<td>1641</td>
</tr>
<tr>
<td>Minimum</td>
<td>1946</td>
<td>1297</td>
</tr>
<tr>
<td>Maximum</td>
<td>8973</td>
<td>12324</td>
</tr>
<tr>
<td>Lower 95% CI of mean</td>
<td>4176</td>
<td>4034</td>
</tr>
<tr>
<td>Upper 95% CI of mean</td>
<td>4730</td>
<td>4717</td>
</tr>
</tbody>
</table>

**Figure 10.** Acetylcholinesterase (AChE) values of all samples by pineapple cultivation areas.
Sporophila americana (Kruskal-Wallis Test)

Volatinia jacarina (Kruskal-Wallis Test)
Figure 11. Acetylcholinesterase (AChE) activity per species in each sampled growing area.
Figure 12. Acetylcholinesterase (AChE) activity (IU/g) in brain tissue by gender, sampled species and the pineapple-growing area. Differences were considered as significant at a level of $p \leq 0.05$ (*).
4.2.3 In vitro inhibition of butyrylcholinesterase activity by carbaryl in plasma from control parrots

The results of the in vitro inhibition assays are presented in Table 7. The calculated percentages after adding 50 µg of carbaryl to the plasma of parrots revealed BChE inhibition rates of 50% and 79% when using Kit 1 and Kit 2, respectively.

Table 7. Results of the in vitro test: inhibition of BChE in plasma of control parrots by carbaryl.

<table>
<thead>
<tr>
<th></th>
<th>Plasma values obtained</th>
<th>Plasma values adding 50 µg carbaryl</th>
<th>Inhibition percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit 1</td>
<td>8525</td>
<td>4603</td>
<td>50%</td>
</tr>
<tr>
<td>Kit 2</td>
<td>1897</td>
<td>416</td>
<td>79%</td>
</tr>
</tbody>
</table>

4.3. Pesticides

Feathers

A total of 207 feather samples were analyzed. A certain concentration of pesticides was found in the extracts of feathers and soil. Generally, external contamination could be proven in the wild birds in all four conventional pineapple-growing areas. Both genders of all four species were affected. Of the 109 birds from the conventional pineapple plantation, 36 showed detectable levels of diuron and 32 of ametryn (Table 8). Considering the four species and the number of collected animals disregarding gender, 22% of Ramphocelus passerinii and Volatinia jacarina, 30% of Sporophila americana and 56% of Sporophila funerea were contaminated with diuron. The following data were observed for ametryn: 22% of Ramphocelus passerinii, 27% of Volatinia jacarina, 26% of Sporophila americana and 43% of Sporophila funerea were
contaminated, regardless of sex. Of a total of 98 feather samples from the organic pineapple-growing areas, diuron (0.602 mg/kg) and ametryn (0.98 mg/kg) were only detected in one animal.
Table 8. Amount of diuron and ametryn (mg/kg) found in each analyzed sample by gender and species

<table>
<thead>
<tr>
<th>Bird species</th>
<th>Gender</th>
<th>Diuron mg/kg</th>
<th>Ametryn mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sporophila funerea</td>
<td>Female</td>
<td>0.558</td>
<td>0.953</td>
</tr>
<tr>
<td>Sporophila funerea</td>
<td>Female</td>
<td>0.138</td>
<td></td>
</tr>
<tr>
<td>Sporophila funerea</td>
<td>Female</td>
<td>0.643</td>
<td>0.765</td>
</tr>
<tr>
<td>Sporophila funerea</td>
<td>Female</td>
<td>1.207</td>
<td>1.603</td>
</tr>
<tr>
<td>Sporophila funerea</td>
<td>Female</td>
<td>0.993</td>
<td>0.408</td>
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<tr>
<td>Sporophila funerea</td>
<td>Female</td>
<td>0.928</td>
<td>0.753</td>
</tr>
<tr>
<td>Sporophila funerea</td>
<td>Female</td>
<td>2.931</td>
<td>2.219</td>
</tr>
<tr>
<td>Sporophila funerea</td>
<td>Male</td>
<td>0.152</td>
<td></td>
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<tr>
<td>Sporophila funerea</td>
<td>Male</td>
<td>0.452</td>
<td>0.562</td>
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<tr>
<td>Sporophila funerea</td>
<td>Male</td>
<td>1.469</td>
<td>1.597</td>
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<tr>
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<td>Male</td>
<td>5.714</td>
<td>1.466</td>
</tr>
<tr>
<td>Sporophila funerea</td>
<td>Male</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>Sporophila funerea</td>
<td>Male</td>
<td>0.981</td>
<td>1.066</td>
</tr>
<tr>
<td>Sporophila americana</td>
<td>Female</td>
<td>5.157</td>
<td>4.409</td>
</tr>
<tr>
<td>Sporophila americana</td>
<td>Female</td>
<td>7.18</td>
<td>19.997</td>
</tr>
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<td>0.795</td>
<td>0.523</td>
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<td>Female</td>
<td>14.599</td>
<td>17.896</td>
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<td>1.982</td>
<td>0.621</td>
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<td>Volatinia jacarina</td>
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</tr>
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<td>Volatinia jacarina</td>
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<td>0.714</td>
<td>1.181</td>
</tr>
<tr>
<td>Volatinia jacarina</td>
<td>Female</td>
<td>0.693</td>
<td>0.733</td>
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<td>Volatinia jacarina</td>
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<td>0.125</td>
<td>0.377</td>
</tr>
<tr>
<td>Volatinia jacarina</td>
<td>Male</td>
<td>6.113</td>
<td>2.906</td>
</tr>
<tr>
<td>Volatinia jacarina</td>
<td>Male</td>
<td>0.47</td>
<td></td>
</tr>
</tbody>
</table>
Skin

The extracts, a total of 31 skin pool samples, were analyzed by the described method, and no pesticide was detected.

Feces

The extracts, a total of 29 feces pool samples, were analyzed by the described method, and no pesticide was detected.

Soil

In the soil samples from the organic fields, no pesticides were found. Different amounts of three very frequently used pesticides were detected in the conventional samples: ametryn and diuron as herbicides and bromacil as an insecticide. Two extracts were prepared and analyzed for each sample (Table 9).

Table 9. Summary of the pesticides detected in the soil samples collected in the conventional pineapple-growing areas.

<table>
<thead>
<tr>
<th>Area</th>
<th>Ametryn (mg/kg)</th>
<th>Diuron (mg/kg)</th>
<th>Bromacil (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 1</td>
<td>0.143</td>
<td>2.731</td>
<td>0.608</td>
</tr>
<tr>
<td>C 1</td>
<td>0.136</td>
<td>2.668</td>
<td>0.601</td>
</tr>
<tr>
<td>C 2</td>
<td>traces</td>
<td>0.085</td>
<td>ND</td>
</tr>
<tr>
<td>C 2</td>
<td>traces</td>
<td>0.073</td>
<td>ND</td>
</tr>
<tr>
<td>C 3</td>
<td>0.046</td>
<td>1.352</td>
<td>0.706</td>
</tr>
<tr>
<td>C 3</td>
<td>0.043</td>
<td>1.116</td>
<td>0.665</td>
</tr>
<tr>
<td>C 4</td>
<td>2.071</td>
<td>6.314</td>
<td>1.019</td>
</tr>
<tr>
<td>C 4</td>
<td>2.048</td>
<td>6.570</td>
<td>1.056</td>
</tr>
</tbody>
</table>
5 Discussion

Environmental observations

Pesticides exert direct and indirect effects on wild birds. The direct ones are associated with an increase in the mortality rates of target bird populations, considered as pests or a danger for a specific crop. For example, granular carbofuran reduced the population of 32 different wild bird species living around rice crop areas in USA and Senegal (FLICKINGER u. KING, 1972; FLICKINGER 1979; MULLIE et al., 1991). In many areas of South America, some organophosphates are used as target-avian pesticides (parathion and monocrotophos), especially in rice fields, even though it is clear that the practice is illegal (BASILII u. TEMPLE, 1999 a, b). However, the four wild bird species analyzed in the present study were not considered as a menace and they were selected only for the detection of environmental contamination with pesticides. Moreover, several field studies are needed to determine the acute or chronic effects related to avian mortality, occurring either sporadically or regularly (FITE et al., 1988; MINEAU, 2002).

The indirect effects are related to the reduction of the prey populations and habitat changes. The prey species for birds include macroinvertebrates (especially insects), amphibians and fishes (PARSONS et al., 2010). Several studies performed in France (TOURENQ et al., 2003; MESLEARD et al., 2005) correlated the intensive soil management and pesticide applications (mainly insecticides) with reduced prey biomass estimations for aquatic birds. According to the environmental behavior observed in the birds analyzed in this study, the high variability of the number of birds of a certain species in a certain area can be also attributed to different feeding habits, seasonal variation of food composition, sampling area, age and health condition of the
birds (GOLDEN u. RATTNER, 2003). This may also be the case of this field study, since even by a thorough observation of the wildlife we were not able to find out for sure, how long exactly each bird was or was not exposed to pesticides and how long it was living in the sample collection area. Furthermore, this investigation only focused on one pineapple plantation in a single Costa Rican region. Further and more detailed investigations are needed for a better evaluation of the impact of pesticides on avian populations as related to the pineapple plantation areas in different regions of Costa Rica.

**Enzyme activity**

Birds appear to be more sensitive to the acute exposure to anticholinesterase pesticides due to a reduced level of anticholinesterases detoxifying enzymes (PARKER u. GOLDSTEIN, 2000). Because of the high activity of AChE in the brain of birds, the binding of organophosphates and carbamates to ACh occurs faster than in other vertebrates (WESTLAKE et al., 1983; HILL, 1992). Wild birds can be chronically exposed to agrochemical contamination when living in resident populations or they can be exposed to pesticides for only several months in the case of migratory populations (PINOWSKI et al., 1991). In this study, these four species were chosen because they are non-migratory wild birds and therefore linked to the ecotoxicological status of the analyzed pineapple cultivation areas. To the best of our knowledge, no studies related to cholinesterase enzyme activity in *Volatina jacarina, Sporophila americana, Ramphocelus passerinii* and *Sporophila funerea* have been published so far.

Blood is the best biological material for non-invasive biomarker analysis (FOSSI et al., 1994). Plasma ChE activity has been used to monitor the exposure to anti-cholinesterase pesticides in many bird species and returns to normal values faster than the ChE of red blood cells
Cholinesterase activity in the blood may be used as an indicator of pesticide presence, since blood ChE activity is more sensitive than brain ChE activity to pesticides (HILL, 1992; FOSSI et al., 1996). In fact, our data in all four wild bird species support the concept that blood ChE activity is a more sensitive parameter than brain ChE activity. Nevertheless, the results obtained in this study also coincide with the observations previously made by many authors suggesting that plasma ChE is a good biomarker at low levels of exposure to organophosphorus pesticides because it is more rapidly inhibited and diminish to a larger extent than brain ChE (HILL u. FLEMING, 1982; SANCHEZ et al., 1997; SOLER-RODRIGUEZ et al., 1998). For example, BChE may be scavenging the active oxon forms of organophosphorus compounds that otherwise might inhibit brain AChE activity (GUPTA u. DETTBARN, 1987).

According to FAIRBROTHER u. BENNETT (1988), one of the major problems in measuring the inhibition of ChE activity in brain tissue is the determination of what represents a “normal” value of ChE activity for a given species and to what extent an individual value may have to be reduced before it becomes indicative of exposure to or death due to a ChE inhibitor.

As this investigation analyzed free-living non-migratory wild birds, no age restrictions were established. Some of the individuals were young birds. Several authors suggest that the BChE activity varies with age, and young birds have a higher enzyme activity when compared to adult birds (LYLES et al., 1980; BENNETT u. BENNETT, 1991; GARD u. HOOPER, 1993).
The highest plasma cholinesterase activity in wild birds has been reported to be measured during the early morning hours (COBOS et al., 2010). This observation justified the time of sample collection in the present study: a high percentage of these four wild bird species were indeed captured in the morning hours.

Hence, variations in the measured BChE and AChE activities may be related to several variables such as species, gender, age, and time of sampling (HILL, 1988; BENNETT u. BENNETT, 1991; FILDES et al., 2009, LAJMANOVICH et al., 2009). The results obtained in this investigation demonstrated that there was a significant difference in BChE activity of birds living in conventional and organic areas. BChE activity seems to be a sensitive marker for environmental monitoring of wild birds in Costa Rica. Using the data generated in our study, a more adequate sample size can now be calculated in order to determine differences among birds which are captured in different cultivation areas.

**In vitro plasma BChE inhibition by carbaryl**

The plasma BChE values obtained in African grey parrots (*Psittacus erithacus*) in the frame of the *in vitro* test before addition of carbaryl coincides with the normal plasma BChE activity range described by KIESAU (1997) for these bird species, thereby justifying the choice of the ChE kits used in the present study and the measurement of the enzyme activity in the absence or presence of inhibiting agents.

**Pesticides in soil samples**

Northern Costa Rica is a region with strong rainfall, which supports the rapid growth of weeds. Therefore, an adequate soil preparation and the consistent and timely application of herbicides
(especially diuron 80% and commercial ametryn products) are required for a suitable weed control in the pineapple plantations (VILLEGAS et al., 2007). Herbicides were the most frequent pesticide group found in bird feathers and soil samples in this study, which is in accordance with the findings made by CASTILLO et al. (1997) in this Costa Rican region.

Concentration levels of diuron, ametryn and bromacil were detected in soil samples collected in each conventional pineapple-growing area, as reported by VARGAS (2010) in soil and subterranean water sources near to pineapple plantations in the Caribbean zone of Costa Rica. In the case of bromacil, high concentrations were measured in water samples from wetlands of Northern Costa Rica by RAMIREZ (2010), exceeding the maximum allowable concentration for protection of the 95% of the species in an ecosystem established by the regulations of the European Union. A direct exposure can occur when mammals and birds get in contact with residues of Krovar® (granulated commercial bromacil) through their skin or eyes or when they inhale vapors or particulates, while indirect exposure may occur when mammals and birds eat contaminated prey or vegetation (USEPA, 2003). However, even though suspected, in this study both exposure routes could not be yet demonstrated.

On the other hand, the use of diuron is forbidden in fields located near rivers and water-logged areas (APVMA, 2012). In Australia, for example, the use of this herbicide is restricted in wet tropical areas (in both pre-emergent and post-emergent periods), including pineapple plantations in Queensland. MORALES-VARGAS (2013) described the presence of diazinon, ametryn and other nitrogen-containing herbicides in alluvial aquifers on the Pacific coast of Costa Rica, which is associated with the sugarcane and melon cultivation. Due to its high persistence (one month to one year), diuron can be found in many environments such as soil, sediments and water (FIELD
et al., 2003; OKAMURA et al., 2003). The direct spray of diuron may represent a risk to insects, birds, and mammals, primarily when the maximum application rate is used (USEPA, 2003b).

Ametryn was the third herbicide compound found in the conventional area soil samples as well as in bird feathers. According to the United States Environmental Protection Agency (USEPA, 2003b), it is expected that the exposure to ametryn pollutes food and forage because the treated fields provide a habitat rich in food sources attractive to various avian and mammalian species. However, at the present time acute exposure to ametryn does not pose a risk for birds.

**Pesticides in feathers**

The highest concentrations of pesticides were found on the feathers. Many authors reported that the potential risk from agrochemicals for wild birds also depends on the formulation and the active ingredient concentration of the discharged pesticide particles and environmental factors such as weather or soil humidity (HILL u. CARMADESE, 1981; BEST u. FISCHER, 1992; STAFFORD u. BEST, 1997). Even though data on wild bird feathers contamination in Costa Rica is still not available, the results of this study can somehow be compared with those of PINNOCK et al. (2014) in sloth hairs from Northeastern Costa Rica. Moreover, MINEAU (2012) described that the pesticide absorption and its relative toxicity via the dermal and oral routes is similar in mammals and birds. In the present study, it has been confirmed that wild bird feathers in the conventional pineapple-growing areas were contaminated by the herbicides diuron and ametryn, indicating that these birds could have been in indirect contact with these pesticides, even though this exposure is not yet taken into account by the pesticide registration authorities, as previously described by DRIVER et al. (1991) and MINEAU (2011). Pesticide residues on
feathers, feet and skin as well as in the gastrointestinal contents have been detected at higher levels in wild Canada goose (*Branta canadensis*) goslings than in goslings used in laboratory dietary toxicity studies (VYAS et al., 2006). This fact justifies the direct collection of the samples in the pineapple growing fields and not to develop an experiment under laboratory conditions.

Very similar observations to those reported in this study were previously described by MORTENSEN et al. (1998) in foot and carcass washes made from birds exposed to propiconazole, carbofuran and ametryn in Costa Rican banana plantations. In accordance with this study, the wild birds were sampled during the dry season, which made possible to find pesticides on their feathers. However, during the wet season, herbicides and insecticides were quickly dissolved and dispersed in water sources and puddles and then finally taken up or absorbed by the birds (MORTENSEN et al., 1998). According to the study by DRIVER et al. (1991) on bobwhite quail (*Colinus virginianus*) and the results obtained by MINEAU (2002), oral acute pesticide intoxication can also occur when the birds brush their plumage, this risk being comparable to dermal intoxication or even the direct intake of poisoned food or water.

Furthermore, differences in the degree of external contamination among different feather types due to differences in the extent of the exposure should also be considered (ALTMEYER, 1991; DAUWE et al., 2003a). The age of the feather may be an important issue, as it will be linked to the time of exposure. For example, it has been shown that adult common buzzards (*Buteo buteo*) do not complete their moult in one season (ZUBEROGOITIA et al., 2005), leading to considerable variation in the age of the feathers. Moreover, the amount of feather samples taken for analysis depends on the concentration of the analyte, the sensitivity of the analytical method, and the number of replicated determinations (BOGDANOV et al., 2006). The size and amount of
feathers were limiting factors in this study. Therefore, it was necessary to apply methods like QuEChERS, which is extremely useful to quantify a bright spectrum of pesticides. Usually, the amount of a hair sample is one to five grams (SCHRAMM, 1999) for the analysis of trace pollutants in the ng/kg range. In some cases, the lowest limit of determination is reported at a level of several pg/mg of the hair and allows the investigation with a single hair (WAINHAUS et al., 1998). Generally, the powder-like state of the sample will increase the efficiency of subsequent extraction (ZHANG et al., 2007a, b).

ALTMEYER et al. (1991) reported that when using feathers as a biomonitor for heavy metal pollution, exact information is required regarding the moulting stage as well as the type and the position of the feathers. In this study, it was not possible to determine how long the birds were exposed to external contamination, and probably further studies on the feather contamination during the rainy season in Costa Rica are required; however, our results support the concept to perform feather studies as a useful tool for the identification of organic pollutants.

**Pesticides on skin**

As the feathers have been proven to be an inadequate barrier against pesticide exposure, skin analyses were also performed (POPE u. WARD, 1972; MINEAU et al., 1990). However, it was not possible to determine the presence of pesticides in the pooled skin samples. The presence of chemicals in the skin causing percutaneous toxicity was not expected, since acute oral toxicity represents a more important route of toxicity, highlighting skin as a very effective barrier (HUDSON et al., 1979).
Even though the present study could not demonstrate the presence of pesticides on the bird skin because of the small amount of the analyzed dermal samples, there is evidence that pesticide dermal exposure in birds has been underestimated for a long time and should be accepted as part of the pesticide risk assessment process (MINEAU, 2012; ALHARBI et al., 2015).

The prevalent application of pesticides throughout the year might cause chronic effects. This observation in the present study agrees with conclusions raised by MINEAU (2002) indicating that bird mortality might be due to the intensive and prolonged use of pesticide sprays.

The toxic effect of a pesticide to birds and the application rate can influence the mortality rate after dermal exposure; however, the influence of other factors such as dermal toxicity and volatility of a pesticide remain unclear up to now (MINEAU, 2002).

A single field study by itself may not be sufficient to dispel the presumption of high risk that is placed on a pesticide. This is because of the stochastic variability encountered in most field situations as well as the inability to detect impacts every time they occur (in part because of the difficulty of discovering evidence of impact in carcasses). This requirement means substantial costs if one wanted to determine whether each pesticide could lead to avian toxicity (essentially mortality) (MINEAU, 2002).
6 Conclusions

1. In the present plasma and brain cholinesterase activities were determined by the Ellmann method in four free-living wild birds in pineapple cultivation areas of Northern Costa Rica. Based on the differences observed among birds captured in conventional and organic pineapple plantations, BChE activity appears to be a sensitive biomarker for future ecotoxicological studies in Costa Rica.

2. The UHPLC-TOF-MC analysis established the presence of bromacil, diuron and ametryn in feather samples of four wild bird non-migratory species (S. americana, S. funerea, V. jacarina and R. passerinni) as well as in soil samples, both obtained from different collection points in Costa Rican conventional and organic pineapple-growing areas. These findings show that there is a need for environmental risk assessment in the case of wild birds living near pineapple plantations of Costa Rica.

3. Since no AChE and BChE values for these tropical wild bird species were available before, it is recommended that further studies be conducted, focusing on the environmental impact of pineapple pesticides on wildlife birds and their habitats and on the search of scientific strategies for sustainable pineapple production.
7 Outlook

The information documented in this doctoral thesis tracked the pesticide contamination after their application in pineapple producing areas. It is postulated that the data obtained in the course of this study could be used in future studies to determine if the use of products for pest control could represent a risk for animal and human health.

The multidisciplinary research team, in which three Costa Rican state universities participated, included biologists, agricultural scientists and social workers. The feedback of the different scientific disciplines was important to better understand the different facets related to the use of the pesticides in Costa Rican pineapple production and their environmental effects. Without this feedback, this doctoral thesis would not have been completed, especially because explicit information regarding the physiology and ecology of the four wild bird non-migratory species (*S. americana, S. funerea, V. jacarina* and *R. passerinii*) analyzed in this study is still limited (for example, the basal metabolic rates (BMR) of these species are still under investigation and there is no data regarding their AChE and BChE activities).

This investigation was performed during the end of the dry season in Costa Rica (March and April 2012) to minimize the temporal variation of the values of the markers, according to various previous studies in birds. Moreover, further studies should be performed at different times of the year to complete and compare the obtained results with new data that could be helpful in the interpretation of chronic effects induced by pesticides in the frame of environmental monitoring.

In summary, it can be said that this thesis forms a solid basis for further research on tropical birds and their relevance as toxicological biomonitors. The experience and data collected in the course
of this study could be useful for future research regarding the detection of environmental pollution in fruit producing areas and may result in a more friendly management of nature, thereby conserving the wildlife diversity.
8 Summary

Angelova, Lora:

Wild birds as a bioindicator for wildlife toxicity in pineapple cultivation areas in Northern Costa Rica

Four non-migratory wild bird species (S. americana, S. funerea, V. jacarina and R. passerinii), inhabiting the gallery forests close to the conventional and organic pineapple-growing areas in Northern Costa Rica were selected to evaluate their exposure to organophosphates and carbamates by measuring plasma and whole brain cholinesterase activities as well as the levels of pesticides accumulating in feather, skin, feces and soil samples by using UHPLC-TOF-MC (Ultra High Performance Liquid Chromatography Time Of Flight Mass Chromatography) analysis. Samples were collected, classified and compared in the following order: 196 plasma (108 conventional and 88 organic), 197 brain (106 conventional and 91 organic), 201 feather samples (109 conventional and 92 organic), 31 pooled skin (109 conventional and 92 organic), 29 pooled feces (109 conventional and 85 organic) and two soil samples from each collection point (four conventional and four organic). Butyrylcholinesterase (BChE) activity in plasma and acetylcholinesterase (AChE) activity in brain were evaluated with the aim of obtaining information on the differences between genders, species and living areas by using Ellmann’s spectrophotometric method and were statistically analyzed by performing the Kruskal-Wallis and the Mann-Whitney tests.

S. americana in the organic pineapple areas has a higher BChE activity than S. americana in the conventional ones. In the case of V. jacarina and R. passerinii, all the activity values of the males were lower than those of the females. The measured enzyme activity of S. funerea males in both
pineapple areas was similar. Generally, all birds captured in the organic pineapple plantations showed higher levels of BChE than those living in the conventional ones. In the case of AChE, there was a statistically significant difference between male and female of *S. americana* in the conventional areas. The *V. jacarina* and *R. passerinii* females showed higher enzyme activities than males, while the opposite was observed in the case of *S. americana* and *S. funerea*. The *R. passerinii* and *S. funerea* males from the organic pineapple cultivation areas showed higher activities than the males from the conventional ones. No differences were found between the AChE values of *V. jacarina* males and females in the conventional areas. Contamination of the feathers by ametryn and diuron was observed in all the species, independently of the gender. Ametryn, diuron and bromacil were detected in the soil of the conventional pineapple cultivation areas that coincides with other studies conducted in different Costa Rican regions. No pesticides were detected in the pooled samples of skin and feces. Neither a direct effect on the health of the birds nor signs of toxicity were observed. However, indirect effects in the bird ecosystems and their feed sources cannot be discarded. Since up to now, no AChE and BChE activity data were available for these tropical wild bird species, it is recommended that further studies are undertaken to determine the environmental impact of pineapple pesticides on wildlife birds and their habitats.
9 Zusammenfassung

Angelova, Lora:

Wildvögel als Bioindikator für Wildtierkontamination in Ananasanbaugebieten in Nord Costa Rica


10 References

ALDRIDGE, W.N. (1953):
The inhibition of erythrocyte cholinesterase by tri-esters of phosphoric acid. 3. The nature of the inhibitory process.

Organophosphate pesticide method development and presence of chlorpyriphos in the feet of nearctic-neotropical migratory songbirds from Canada that over-winter in Central America agricultural areas.
Chemosphere. 27, 827-835.

Plasma and whole brain cholinesterase activities in three wild bird species in Mosul, Iraq: In vitro inhibition by insecticides.
Interdiscip. Toxicol 4,144-148.

ALLES, G.A. u. R.C. HAWES (1940):
Cholinesterases in the blood of man.


AUGUSTINSSON, K.B. (1949):
Substrate concentration and specificity of choline ester-splitting enzymes.
Arch. Biochem. 23, 111 – 126

AUSTRALIAN PESTICIDES AND VETERINARY MEDICINE AUTHORITY (AVPMA).
(2012):
Diuron Chemical Review-9 Implementation.
Symonston, Australia.

BACEY, J. (2000):
Environmental Fate of Hydramethylnon;
California Environmental Protection Agency, Department of Pesticide Regulation. Available at

Pesticides.
In: Immunotoxicology and Immunopharmacology.
BASILI, G.D. u. S.A. TEMPLE (1999a):
Dickcissels and crop damage in Venezuela: defining the problem with ecological models.
Ecol. Appl. 9, 732-739.

Winter ecology, behavior, and conservation needs of Dickcissels in Venezuela.
Studies in Avian Biology 19, 289-299.

BAYER CROPSCIENCE® CANADA (2012):
Chipco® Sevin® T&O Carbaryl Insecticide- Material safety data sheet

BEAVERS, J. (1990):

Biomonitoring with birds
In: Bioindicators and biomonitors.
Age-dependent changes in activity of mallard plasma cholinesterases.
J. Wildlife. Dis. 27, 116-118.

Effects of dietary exposure to methyl parathion on egg laying and incubation in mallards.

Granular insecticides and birds: Factors to be considered in understanding exposure and reducing risk.
Environ. Toxicol. Chem. 11, 1495 - 1508

BLI (BIRD LIFE INTERNATIONAL) (2008):
State of the World’s birds: Indicators for our changing world.
Bird Life International, Cambridge, UK.

Characterization and assay conditions for use of AChE activity from several marine species in pollution monitoring.
Determination of organic compounds in human hair.
Anal. Chem. 61, 936–951.

BOONE, J.S. u. J.E. CHAMBERS (1997):
Biochemical factors contributing to toxicity differences among chlorpyriphos, parathion and methyl parathion in mosquitofish (Gambusia affinis).

Hair as a biological indicator of drug use, drug abuse or chronic exposure to environmental toxicants.
Int. J. Toxicol. 25, 143-163.

BRADFORD, M.M. (1976):
A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.
BRAVO, V. (2007):
Diagnosis of pesticide use in banana: Atlantic region Costa Rica.
IRET-UNA, Heredia, Costa Rica.

Monitoring pesticide use and associated health hazards in Central America.

BRITISH CROP PROTECTION COUNCIL (2000):

BURGER, J. (1993):
Metals in avian feathers: bioindicators of environmental pollution.
Reviews in Environ. Toxicol. 5, 203-311.

Ecotoxicology and pesticides in tropical aquatic ecosystems of Central America.
Environ. Toxicol. Chem. 16, 41-51.
Plaguicidas y otros contaminantes.
18º Informe Estado de La Nación en Desarrollo Humano Sostenible. San José, Costa Rica.

Effect of a single dose of an acetylcholinesterase inhibitor on oxotremorine- and nicotine-induced hypothermia in mice.
Phamacol. Biochem. Behav. 39, 929-934

COMMITTEE ON FOOD CHAIN AND ANIMAL HEALTH (CFCAH) - EUROPEAN UNION COMMISSION (2010):
Final review report for the active substance metalaxyl.

COVACI, A. u. P. SCHEPENS (2001b):
Chromatographic aspects of the analysis of selected persistent organochlorine pollutants in human hair.
Hair analysis: another approach for the assessment of human exposure to selected persistent organochlorine pollutants. 
Chemosphere 46, 413-418.

Organochlorine pollutants in human hair. 
J Anal Toxicol. 22, 610-611.

Variation of heavy metals within and among feathers of birds of prey: effects of molt and external contamination. 
Environ. Pollut. 124, 429-436.

DAUWE, T., JASPERS, V., COVACI, A., SCHEPENS, P. u. M. EENS (2005): 
Feathers as a nondestructive biomonitor for persistent organic pollutants. 
Environ Toxicol Chem 24, 442-449.

DE VOS, S. u. R. DE SCHRIJVER (2005): 
Polychlorinated biphenyl distribution and faecal excretion in rats fed wheat bran. 
Chemosphere 61, 374-382.
Hair as an indicator of endogenous tissue levels of brominated flame retardants in mammals.

Non-destructive exposure and risk assessment of persistent pollutants in the European hedgehog
(Erinaceus europaeus).

D.B., DROWN (1991):
Routes of uptake and their relative contribution to the toxicological response of northern
bobwhite (Colinus virginianus) to an organophosphate pesticide.

Unpublished study prepared by Hazleton Laboratories America, Inc.

A new and rapid colorimetric determination of acetylcholinesterase activity.


Unpublished study prepared by Hill Top Research, Inc. pp. 29.

FAIRBROTHER, A. (1996):

Cholinesterase-inhibiting pesticides.

In: Non infectious Diseases of Wildlife


FAJGELJ, A. u. Á. AMBRUS (2000):

Principles and Practices of Method Validation.


FAO (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS) (2011):

Pineapple production in Costa Rica in 2009.

FAO (FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS) (2013):
Fosetyl-Aluminum: Aluminum tris (ethylphosphonate)

Cholinesterase response in native birds exposed to fenitrothion during locust control operations in eastern Australia.
Environ Toxicol Chem. 25, 2964-2970.

Plasma cholinesterase characteristics in native Australian birds: significance for monitoring avian species for pesticide exposure.
Austral ornithology. 109, 41-47.

FINK, R. (1976):
FINK, R., BEAVERS, J.B. u. R. BROWN (1978):
Unpublished study prepared by Wildlife International, Ltd.

Hazard evaluation division, standard evaluation procedure: Guidance document for conducting terrestrial field studies.
Environmental Protection Agency 540/09-88/109. Washington, DC, USA.

Arch Environ Contam Toxicol. 46, 542-550.

FLECHTER, D. u. C. PEDERSEN (1988a):
Diazinon MG8 Technical: 14-Day Acute Oral LD50 Study in Mallard Ducks: Project ID: BLAL No. 88 DD 56.
FLECHTER, D. u. C. PEDERSEN (1988b):
Diazinon MG8 Technical: 14-Day Acute Oral LD 50 Study in Brown-headed Cowbirds: Project ID:
BLAL No. 88 SB 103.

FLICKINGER, E.L. u. K. A. KING (1972):
Some effects of aldrin-treated rice on Gulf Coast wildlife.
J. Wildl. Manage. 36, 706-727.

FLICKINGER, E.L. (1979):
Effects of aldrin exposure on Snow Geese in Texas rice fields.
J. Wildl. Manage. 43, 94-101.

The use of non-destructive biomarkers in the hazard assessments of vertebrate populations.
In: Nondestructive Biomarkers in Vertebrates.

FOSSI, M. C., LARI, L. u. S. CASINI (1996):
Interspecies variation of ‘B’ esterases in birds: the influence of size and feeding habits.
Arch Environ Con Tox 31, 525–532.

Effects of exposure to an organophosphorus pesticide on the behavior and use of cover by captive starlings.

Environ Toxicol Chem 15, 1590–1596.


Birds as monitors of pollutants.

_In:_ Birds as monitors of environmental change.


Circadian rhythms of determined blood chemistry values in buzzards and eagle owls.

Comp. Biochem. Physiol. 88, 663–669.


Age-dependent changes in plasma and brain cholinesterase activities of Eastern bluebirds and European starlings.

Ranking terrestrial vertebrates species for utility in biomonitoring and vulnerability to environmental contaminants.

Monitoring and assessment of Swainson’s hawks in Argentina following restrictions on monocrotophos use, 1996-1997.
Ecotoxicology 8, 215-224.

Diazinon MG8: A comparison of dietary LC50 values with mallards of different ages.
Wildlife International Ltd. Project No. 109278.


Oxamyl: A Dietary LC50 Study with the Bobwhite
Unpublished study prepared by Wildlife International Ltd. 26 p.
Interpreting population estimates of birds following pesticide application-behavior of male starling exposed to an organophosphate pesticide.

Response of common grackles to dietary concentrations of four organophosphate pesticides.
Arch. Environ. Contam. Toxicol. 11, 617-626.

Sensitivity of nestling and adult starlings to dicrotophos, an organophosphate pesticide.

Biological Consequences of Depressed Brain Cholinesterase Activity in Wildlife.
In: Cholinesterase Inhibiting Insecticides

Neurophysiological and behavioral changes in non-target wildlife exposed to organophosphate and carbamate pesticides thermoregulation food consumption and reproduction.
Am Zool 37, 369–388.

GUILHERMINO, L. (2007):

The use of biomarkers to assess the effects of environmental contamination in coastal and estuarine ecosystems: what questions remain? An example of the Portuguese NW coast.
International Council for the Exploration of the Sea Conference Meeting (ICES-CM), 1, 17.


Iso-OMPA-induced potentiation of soman toxicity in rat.
Arch. Toxicol. 61, 58-62.

HART, A. D. M. (1993):

Relationships between behavior and the inhibition of cholinesterase in birds exposed to organophosphorus pesticides.
The Agrochemical Handbook.
Nottingham, England: Royal Society of Chemistry.

Brain Cholinesterase activity of apparently normal wild birds

Avian toxicology of anticholinesterases.
In: Clinical and Experimental Toxicology of Organophosphates and Carbamates.

Organophosphorus and carbamate pesticides.
In: Handbook of Ecotoxicology
Wildlife Toxicology of Organophosphorus and Carbamate Pesticide.

In: Handbook of Ecotoxicology.


Subacute toxicity testing with young birds: response in relation to age and interest variability of LC$_{50}$ estimates.

In: Avian and Mammalian Wildlife Toxicology: Second Conference,

Anticholinesterase poisoning of birds: field monitoring and diagnosis of acute poisoning.

Environ. Toxicol. Chem. 1, 27-38

Lethal Dietary Toxicities of Environmental Pollutants to Birds: Special Scientific Report
Guidelines for in-house validation of analytical methods for pesticide residues in food and animal feed.
Analyst. 124, 953-958.

Relationship between brain and plasma carbaryl levels and cholinesterase inhibition.
Toxicology 276, 172-183.

Organochlorines, organobromines and their metabolites in eggs of Norwegian birds of prey.
Organohalogen Compd 61, 466-469.

Organophosphorus insecticide exposure in hawks inhabiting orchards during winter dormant-spraying.
Bull Environ Contam Toxicol 42, 651-659.

HOOPER, M.J. (2002):
Swainson’s hawks and monocrotophos, Texas.
HOWARD, P. H. (1991):

Handbook of Environmental Fate and Exposure Data for Organic Chemicals.


Acute oral and percutaneous toxicity of pesticides to mallards: correlations with mammalian toxicity data.
Toxicology and Applied Pharmacology 47, 451-460.


The importance of exogenous contamination on heavy metal levels in bird feathers. A field experiment with free-living great tits, Parus major.
J Environ Monit 6, 356-360.


Can predatory bird feathers be used as a non-destructive biomonitoring tool of organic pollutants?
Heavy metals and selenium in feathers of great tits (*Parus major*) along a pollution gradient.
Environ. Toxicol. Chem. 20, 2815-2820.

JIMENÉZ, M. u. K. SCHOSINSKY (2000):
Valores de referencia de colinesterasa plasmática y eritrocítica en población costarricense: comparación del desempeño clínico de ambas enzimas.
Rev Costarric Cienc Méd. 21, 117-126.

The study of interactive effects of pesticides in birds – a biomarker approach.

Comparative diazinon toxicity in guppy and zebrafish: Different role of oxidative metabolism.
Environ. Toxicol. Chem. 12, 1243-1250.

Royal Society of Chemistry Information Services, Cambridge, UK.
KIESAU, B. (1997):
Untersuchung zur Cholinesterase-Aktivität im Plasma und Gehirn bei Zier-und Wildvögeln.
Dissertation B6522/2. Klinik für Geflügel, Tierärztlichen Hochschule Hannover.

KUENZEL, W. J. (1994):
Central neuroanatomical systems involved in the regulation of food intake in birds and mammals.
J. Nutr. 124, 1355-1370.

KUMAZAWA, T. u. O. SUZUKI (2000):
Separation methods for amino group possessing pesticides in biological samples.

KUTTY, K.M. (1980):
Biological function of cholinesterase.

LAM, P., RICHARDSON, B. u. R. WU (1999):
Introduction to ecotoxicology.
Des Connell, School of Public Health, Griffith University, Queensland, Australia.
Inhibition and recovery of cholinesterases in *Odontophrynus americanus* tadpoles exposed to fenitrothion.

LEHOTAY, S. (2004):
Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) Approach for Determining Pesticide Residues.
*In:* Pesticide Analysis in Methods in Biotechnology.
Vidal Martinez, J. L. & Garrido Frenich, A., eds. (Humana Press, USA).

Validation of a fast and easy method for the determination of more than 200 pesticide residues in fruits and vegetables using gas and liquid chromatography and mass spectrometric detection.
J. AOAC Int. 88, 595-614.

Use of buffering to improve results of problematic pesticides in a fast and easy method for residue analysis of fruits and vegetables.
J. AOAC Int. 88, 615-629.

Evaluation of two fast and easy methods for pesticide residue analysis in fatty food matrixes.

J. AOAC Int. 88, 630–638.

Pesticide poisoning in gulls.

LEITCHER, R.J., KLA;SSON-WEHLER, E. u. A. BERGMAN (2000):
Methyl sulfone and hydroxylated metabolites of polychlorinated biphenyls.
In: The handbook of environmental chemistry.
Paasivirta, J., ed. (Springer-Verlag, Berlin-Heidelberg, Germany)

Higher brominated diphenyl ethers and hexabromocyclododecane found in eggs of peregrine falcons (Falco peregrinus) breeding in Sweden.
Environ Sci Technol 38, 93-96.

The fate of organic xenobiotics in aquatic ecosystems: Quantitative and qualitative differences in biotransformation by invertebrates and fish.
Comp. Biochem. Physiol Part. A. Mol. Integr. Physiol. 120, 43-49.
Changes in levels and forms of cholinesterases in blood plasma of normal and dystrophic chickens.
J. Neurochem 34, 978-987.

LOVELL, J. B. (1979):
Amidinoxyrazones – A New Class of Insecticides.

MADISON, W.I. (1993):
U.S. Fish and Wildlife Services, National Wildlife Health Research Center.

Influence of methyl parathion on gametogenic and acetylcholinesterase activity in the testis of whitethroated munia (Lonchura marabanica).
Arch. Environ. Contam. Toxicol. 30, 384-389.
Testicular functions and serum titers of LH and testosterone in methyl parathion-fed roseringed parakeets.
Ecotoxicol. Environ. Saf. 71, 236-244.

Diazinon: A One-Generation Reproduction Study with the Northern Bobwhite (Colinus Virginianus):
Lab Project Number: 108/292.

Acute carbofuran exposure and cold stress: Interactive effects in mallard ducklings.

“Simplified procedures for the analysis of polycyclic aromatic hydrocarbons in water, sediments and mussels”.
J. Chromatogr. A. 1047, 181-188.

MASON, T.J. u. Y. ZHAO (1994):
Enhanced extraction of tea solids using ultrasound.
Ultrasonics 32, 375-377.
The uses of ultrasound in food technology.
Ultrason. Sonochem. 3, 253-260.

Age-dependent changes in plasma and brain cholinesterase activities of house wrens and European starling.
J. Wildlife Dis. 39, 627-637.

MENDEL, B. u. H. RUDNEY (1943):
Biochem J. 37, 59-63.

MESLEARD, F., GARNERO, S., BECK, N. u. E. ROSECCHI (2005):
Uselessness and indirect negative effects of an insecticide on rice field invertebrates.
Comptes Rendus Biologies 328, 955-962.

An improved method to study the impact of pesticides sprays on a small song bird.
MINEAU, P. (2002):
Estimating the probability of bird mortality from pesticide sprays on the basis of the field study record.
Environ. Toxicol. Chem. 21, 1497-1506.

MINEAU, P. (2011):
Barking up the wrong perch: why we should stop ignoring non dietary routes of pesticide exposure in birds.

MINEAU, P. (2012):
A comprehensive re-analysis of pesticide dermal toxicity data in birds and comparison with the rat.

MITRA, A., CHATTERJEE, C. u. F. MANDAL (2011):
Syntetic chemical pesticides and their effects on birds.
Res. J. Environ. Toxicol. 5, 81-96.
MORALES-VARGAS, R. (2013):
Risk analysis methodology for agrochemical contamination: San Blas River Basin, Costa Rica.

Avian Exposure to Pesticides in Costa Rican Banana Plantations.
Bull. Environ. Contam. Toxicol. 60, 562-568.

Time-course, dose-response and age comparative sensitivity of N-methyl carbamates in rats.
Toxicol. Sci. 114, 113-123.

Impact of furadan 3G (Carbofuran) applications on aquatic macroinvertebrates in irrigated rice in Senegal.
Arch. Environ. Contam. Toxicol. 20, 177-182.
MYERS, D. K. (1953):
Studies on cholinesterase 9: Species variation in the specificity pattern of the pseudo cholinesterases.
Biochem J. 55, 67–79.

NACHMANSOHN, D. u. M.A. ROTHENBERG (1945):
Studies on cholinesterase. On specificity of enzyme in nerve tissue.
J. Biol. Chem. 158, 653–666.

Low acute exposure to organophosphate produces long-term changes in bird feeding behavior.
Ecol. Appl. 9, 1039-1049.

OBAINEH, M. O. u. A. O. MATTHEW (2009):
Toxicological effects of chlorpyrifos and methidathion in young chickens.

Effects of water pollutants and other chemicals on fish acetylcholinesterase (in vitro).
Environ. Res. 21, 327-335.

Characterization of plasma cholinesterase from the White stork (*Ciconia ciconia*) and its in vitro inhibition by anticholinesterase pesticides.


Toxicology.
Williams and Wilkins, Philadelphia, PA, USA: 231.

PADILLA, S., MARSHALL, R.S., HUNTER, D.L. u. A. LOWIT (2007):
Time course of cholinesterase inhibition in adult rats treated acutely with carbaryl, carbofuran, formetanate, nethomyl, methiocarb, oxamyl or propoxur.

The toxicity of some organic herbicides to cattle, sheep and chickens.
Differential toxicities of organophosphate and carbamate insecticides in the nestling European
starling (*Sturnus vulgaris*).

Monitoring wading bird exposure to agricultural chemicals using serum cholinesterase activity.
Environ. Toxicol. Chem. 19, 1317-1323.

Effects of pesticide use in rice fields on birds.
Waterbirds 33, 198-218.

8-Day Acute Dietary LC$_{50}$ Study with Chlorflurenol ME Methyl in Bobwhite Quail: Lab Project
Number: 152-001-01.
Environmental and food applications of LC-tandem mass spectrometry in pesticide-residue analysis: An overview.
Mass Spectrom Rev. 23, 45-85.

Influence of feeding habitat on prey capture rate and diet composition of White Stork (Ciconia ciconia).

Pesticide exposure on sloths (Bradypus variegatus and Choloepus hoffmanni) in an agricultural landscape of Northeastern Costa Rica.
Jour. Environ. Biol. 35, 29-34.

POPE, G.G u. P. WARD (1972):
The effects of a small application of an organophosphorus poison, fenthion on a weaver bird 
(Quelea quelea).
Pestic. Sci. 3, 197-205.

PROSSER, D. u. A. D. HARD (2005):
Assessing potential exposure of birds to pesticide-treated seeds.
Ecotoxicology 14, 679-691.

Pesticide Use in Australia.
Australian Academy of Technological Sciences and Engineering (ATSE).

Avian exposure to organophosphorus and carbamate pesticides on a coastal South Carolina golf 
course.

RAMÍREZ, F. (2009):
Problemática de la expansión piñera en Costa Rica.
In: Red de Acción en Plaguicidas y sus Alternativas para América Latina.
Revista Enlace 86, 6-8.
Technical Reports Series, Instituto Regional de Estudios en Sustancias Tóxicas, Universidad Nacional (IRET-UNA), Heredia Costa Rica.

RAMÍREZ, F. (2010):
Uso de Plaguicidas en el cultivo de piña en la zona Huetar Norte.
Instituto Regional de Estudios en Sustancias Tóxicas, Universidad Nacional (IRET-UNA), Heredia Costa Rica.

Avian endocrine responses to environmental pollutants.

Biological variability and the influence of stress on cholinesterase activity.
In: Cholinesterase inhibiting insecticides, their impact on wildlife and the environment. Chemicals in agriculture Vol. 2.
Black-bellied whistling duck (*Dendrocygna autumnalis*) brain cholinesterase characterization and diagnosis of anticholinesterases pesticide exposure in wild populations from Mexico.  

Diagnóstico de la cuenca de río Frío, Arenal - Huetar Norte, Costa Rica.  
Universitat Autonòma de Barcelona, Spain.

The Effects of Dietary Inclusion of Oxamyl on Reproduction in the Bobwhite Quail.  
Unpublished study; prepared by Huntingdon Research Centre, UK.

RODUSHKIN, I. u. M.D. AXELSSOM (2000):  
Application of double focusing sector field ICPMS for multielemental characterization of human hair and nails. Part II. A study of the inhabitants of northern Sweden.  
SÁNCHEZ, J.C., FOSSI, M.C. u. S. FOCARDI (1997):
Serum “B” estereases as a nondestructive biomarker for monitoring the exposure of reptiles to organophosphorus insecticides.
Ecotoxicol. Environ. Saf. 37, 45-52.

SANDOVAL, A.C.C. (2009):
Insensatez piñera (Foolish pineapple production).
El Financiero (newspaper), San José, Costa Rica. June 21

Biomonitoring Ausgewählter Organischer Chemikalien mit Haaren.
Herbert Utz Verlag GmbH, München, Germany.

Ecosystem consequences of bird declines.

Kinetic and physiochemical properties of brain acetylcholinesterase from the peacock bass (Cichla ocellaris) and in vitro effect of pesticides and metal ions.
Aquat. Toxicol. 126, 191-197.
SINGH, P. u. S.I. RIZVI (2013):
Curcumin Activates Erythrocyte Membrane Acetylcholinesterase.

SMITH, J.D. (1987):
Pesticide use and toxicology in relation to wildlife: organophosphorus and carbamate compounds.

Contaminant exposure in terrestrial vertebrates.
Review. Environ. Poll. 150, 41-64.

Recovery of brain acetylcholinesterase and plasma cholinesterase activities in quail (Coturnix coturnix) after chlorpyriphos administration and effect of pralidoxime treatment.
Manual de recomendaciones para el manejo de la mosca del establo Stomoxys calcitrans en el cultivo de piña.
Instituto Nacional de la Innovación y Transferencia Tecnológica Agropecuaria de Costa Rica (INTA-CR), San José, Costa Rica.

Effects of granular pesticide formulations and soil moisture on avian exposure

STEDMAN, E., STEDMAN, E. u. L.H. EASSON (1935):
Choline-esterase. An enzyme present in the blood-serum of the horse.
Biochem J. 26, 2056–2066.

Guía de aves de Costa Rica.

SVENSMARK, O. (1963):
Enzymatic and molecular properties of cholesterases in human liver.
Agents acting at the neuromuscular junction and autonomic ganglia.

_In: Pharmacological Basis of Therapeutics._

Avian esterases as indicators of exposure to insecticides. The factor of diurnal variation.
_Bulletin of Environmental Contamination and Toxicology_ **4**, 4–11.

Blood esterases as indicators of exposure to organophosphorus and carbamate insecticides.
_In: Nondestructive Biomarkers in Vertebrates._

TOMLINSON, G., MUTUS, B. u. I. MCLENNAN (1980):
Modulation of acetylcholinesterase activity by peripheral site ligands.

Determination of cholinesterase activity in brain and blood samples using a plate reader.
_J. AOAC. Int._ **77**, 1308-1313.
Effects of cropping practices on the use of rice fields by waterbirds in the Camargue, France.
Agriculture, Ecosystems and Environment 95, 543-549.

Washington, DC, USA. pp: 8-32.

U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA) (1992):
Registration Eligibility Decision (RED): Ethylene; EPA-738-F-92-012

U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA) (1998a):
Registration Eligibility Decision (RED): Hydramethylnon; EPA 738-R-98-023.
U.S. Environmental Protection Agency, Office of Pesticide Programs, U.S. Government Printing
Office: Washington, D.C., USA.

U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA) (1998b):
Pesticide Fact Sheet: Metalaxyl.
U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA) (2000):
Pesticide ecotoxicity database on monoethyl ester phosphonic acid aluminum salt (39148-24-8).

U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA) (2003):
Interim Re-registration Eligibility Decision (IRED) for Carbaryl.

U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA) (2006):
Addendum to the 2001 Ethoprop Interim Re-registration Eligibility Decision (IRED)

U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA) (2007a):
Reregistration Eligibility Decision for Oxamyl
U.S. ENVIRONMENTAL PROTECTION AGENCY (USEPA) (2007b):
Reregistration Eligibility Decision for Chlorflurenol Methyl Ester

Hazardous Substances Databank.

Hazardous Substance Data Bank.

Pesticide prioritization for a case-control study on childhood leukemia in Costa Rica: a simple stepwise approach.
Environ Res. 97, 335-347.

Experimental evaluation of the usefulness of feathers as a nondestructive biomonitor for polychlorinated biphenyls (PCBs) using silastic implants as a novel method of exposure.

Environ. Int. 33, 257-264.

VARGAS, I. (2010):

Estudio técnico sobre la existencia o no de contaminación de las aguas con plaguicidas, en la finca Agro Industrial Tico Verde, S.A.


VENAKATESWARLU, K., CHENDRAYAN, K. u. N., SETHUNATHAN (1980):

Persistence and Biodegradation of Carbaryl in Soils.


VERREAULT, J., LETCHER, RJ., MUIR, DCG., CHU, S., GEBBINK, W.A. u. G.W. GABRIELENSEN (2005):

New organochlorine contaminants and metabolites in plasma and eggs of glaucous gulls (Larus hyperboreus) from the Norwegian arctic.

Environmental Toxicology and Chemistry 24, 2486-2499.
VIAL, T., NICOLAS, B. u. J. DESCOTES (1996):
Clinical Immunotoxicology of pesticides.

Changes induced by malathion, methylparathion and parathion on membrane lipid physicochemical properties correlate with their toxicity.

VILLAIN, M., CIRIMELE, V. u. P. KINTZ (2004):
Hair analysis in toxicology.
Clinical Chemistry and Laboratory Medicine 42, 1265-1272.

Caracterización y plan de acción para el desarrollo de la agrocadena de piña en la Región Huetar Norte.
Ministerio de Agricultura y Ganadería (MAG), Ciudad Quesada, Alajuela, Costa Rica.
Immunotoxicology of pesticides: A review.

Oregon State University Extension Pesticide Properties Database.
Oregon State University Extension Service: Corvallis, OR.

Acephate affects migratory orientation of the white-throated sparrow (Zonotrichia albicollis).

Field evaluation of an avian risk assessment model.
Environ. Toxicol. Chem. 25, 1762-1771.

Fast analysis of drugs in a single hair.

Phylogenetic distribution of cholinesterases and related esterases.


Principles of ecotoxicology.
Taylor & Francis, London, UK.

WASHINGTON STATE DEPARTMENT OF TRANSPORTATION (2006):
Bromacil

Pesticide properties database for environmental decision making.
WEED SCIENCE SOCIETY OF AMERICA (1994):
Herbicide Handbook,

WESSELS, J.S.C u. R. VAN DER VEEN (1975):
The action of some derivatives of phenylurethan and of 3-phenyl-1,1 dymethylurea on the Hill reaction.
Biochim Biophys Acta: 19, 548-549.

Organophosphorus poisoning. Effects of selected organophosphate pesticides on plasma enzymes and brain esterases of Japanese quail (Coturnix coturnix japonica).
J. Agric. Fd. Chem. 29, 772 -778.

Carbamate poisoning. Effects of selected carbamate pesticides on plasma enzymes and brain esterases of Japanese quail. quail (Coturnix coturnix japonica).
J. Agric. Fd. Chem. 29, 779 -785.
Control enzyme levels in the plasma, brain and liver from wild birds and mammals in Britain.

Investigation of the significance of heavy metal contents of blackbird feathers.

Avian anesthetics, analgesics and tranquilizers.
Seminars in Avian and Exotic Pet Medicine 2, 7-12.

WILSON, B.W., HOOPER, M.J., LITTRELL, E.E., DETRICH, P.J., HANSEN, M.E.,
Orchard dormant sprays and exposure of red-tailed hawks to organophosphates.
Bull Environ. Contam. Toxicol. 47, 717-724.

Reactivation of organophosphorus inhibited AChE with oximes.
In: Organophosphates Chemistry, Fate and Effects.
WILSON, B.W., MCCURDY, S.A., HENDERSON, J.D., MCCARTHY, S. A. u. J.E. BILLITI.
(1998):
Cholinesterases and agriculture: Humans, laboratory animals, wildlife.
*In:* Structure and function of cholinesterases and related proteins.

Monitoring cholinesterases to detect pesticide exposure.

Automated procedure for estimation of blood cholinesterase activities in rabbits.

ZINKE, A. (2000):
Blutchemische Diagnostik der Organophosphat- und Carbamat-Vergiftungen einschließlich Cholinesterase-Reaktivierung bei Haustauben (*Columba livia v. domestica*).
Thesis, Tierärztliche Hochschule Hannover, Germany.

ZENKER, M. (2004):
Ultraschall-kombinierte Prozessführung bei der Pasteurisierung und Sterilisierung flüssige Lebensmittel.
Thesis. TU Berlin, Germany.

Human hair as a potential biomonitor for assessing persistent organic pollutants.
Environ. Int. **33**, 685–693.

Neutron activation analysis of organohalogens in Chinese human hair.

Sexing, ageing and moult of Buzzards *Buteo buteo* in a southern European area.
Ringing & Migration **22**, 153-158.
11 Acknowledgement

I would like to express the deepest appreciation to Professor Dr. Pablo Steinberg, who gave me his guidance, continuous help and immense knowledge to make possible this dissertation.

I thank to Carlos Luna-Tortós Ph.D. for offering me this interesting project and having faith in my abilities as a professional.

Special thanks to Dr. Michael Empl for his friendly assistance and discussions of various problems.

Also I gratefully acknowledge Liliana Campos for her wisdom, knowledge and commitment to the highest standards and her friendship, which motivated me in some hard moments during this study.

I warmly thank my mother and my brother for their financial and moral support in all aspects of my life. I appreciate as well the understanding and the help of my mother and father-in-law.

I give great thanks to my husband Rodolfo. Without his love, support and encouragements I could not finished this work.