Ist β-Glucosidase ein Schlüsselenzym für die Anpassung an Iridoidglycoside? Ein Vergleich zwischen herbivoren Generalisten (Arctiidae, Lepidoptera) und einem Iridoidglycosidspezialist (Nymphalidae, Lepidoptera)

Dissertation

zur Erlangung der Doktorgrades
der Naturwissenschaften (Dr. rer. nat.)
im Department Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg

vorgelegt von
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aus Freiburg im Breisgau

Hamburg 2010

Genehmigt vom Department Biologie
der Fakultät für Mathematik, Informatik und Naturwissenschaften
an der Universität Hamburg
auf Antrag von Frau Professor Dr. S. DOBLER
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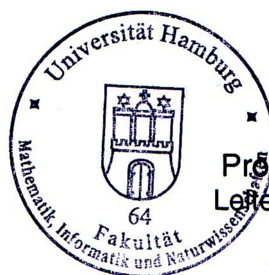
Tag der Disputation: 09. April 2010

Hamburg, den 23. März 2010



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Tag der Disputation: 9. April 2010

Bestätigung zur sprachlichen Korrektheit der in englischer Sprache abgefassten Dissertation

Die sprachliche Korrektheit der in englischer Sprache abgefassten Dissertation
von Frau Helga Carola Pankoke mit wurde von der Unterzeichnenden geprüft und bestätigt.

(*engl.* „The grammatical and linguistical correctness of the dissertation, which has been written in English by Ms. Helga Carola Pankoke, has been proved and confirmed.“)

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Zusammenfassung

Iridoidglycoside sind weit verbreitete pflanzliche Abwehrstoffe, die im Sekundärstoffwechsel vieler Familien der Asteridae synthetisiert werden. Auf nicht angepasste herbivore Insekten wirken Iridoidglycoside einerseits fraßabschreckend und andererseits verlängern sie die Larvalentwicklung durch reduzierte Wachstumsraten und erhöhen die Mortalität. Insekten hingegen, die auf iridoidglycosidhaltige Wirtspflanzen spezialisiert sind, nutzen Iridoidglycoside als Eiablage- oder Fraßstimulantien und einige sequestrieren Iridoidglycoside zum Schutz gegen eigene Fraßfeinde. Bislang ist nur wenig darüber bekannt, wie es herbivoren Insekten möglich ist, die Toxizität von Iridoidglycosiden zu umgehen und iridoidglycosidhaltige Pflanzen ohne schädliche Effekte als Wirtspflanzen zu nutzen.

In der Regel bestehen Iridoidglycoside aus einem Monoterpengrundgerüst, das durch eine β-glucosidische Bindung kovalent mit D-Glucose verbunden ist. Erst die Hydrolyse der β-glucosidischen Bindung führt zur Aktivierung der Iridoide und damit zur Toxizität: Die freigesetzten Iridoidaglykone sind hochreaktiv und können an nucleophile Seitenketten von Proteinen binden, wodurch die Proteine denaturiert und so die Verfügbarkeit und Qualität der Proteine für die herbivoren Konsumenten verringert werden. β-Glucosidase kommt bei Insekten hauptsächlich als Verdauungsenzym vor und spaltet dort von unterschiedlichen Substraten wie z. B. von toxischen pflanzlichen Glycosiden Glucose ab. Aufgrund der spezifischen Art der Aktivierung von Iridoidglycosiden durch β-Glucosidase liegt die Vermutung nahe, dass die β-Glucosidase herbivorer Insekten einen entscheidenden Einfluss auf die Nutzung von iridoidglycosidhaltigen Wirtspflanzen haben könnte.

In der vorliegenden Arbeit untersuche ich die Fragestellung, ob β-Glucosidase ein Schlüsselenzym für die Anpassung an iridoidglycosidhaltige Pflanzen darstellt. Hierfür wurden drei polyphage Bärenspinnerarten (Arctiidae, Lepidoptera) und ein monophager Iridoidglycosidspezialist (Nymphalidae, Lepidoptera) ausgewählt, die unterschiedlich gut an Iridoidglycoside angepasst sind. Alle vier Arten wurden zunächst auf verschiedenen Wirtspflanzen gehalten, die sich hinsichtlich der Konzentration und Zusammensetzung ihrer Inhaltsstoffe unterscheiden: *Taraxcum officinale* (keine Iridoidglycoside), *Plantago major* (geringe Mengen an Iridoidglycosiden) und *P. lanceolata* (hohe Mengen an Iridoidglycosiden). Für den Generalisten *Grammia incorrupta* wurde zusätzlich über die gesamte Larvalphase die Gewichtszunahme in Abhängigkeit von der Futterpflanze bestimmt.

Für die drei Nahrungsgeneralisten *G. incorrupta, Spilosoma virginica,* und *Estigmene acraea* (Arctiidae) sowie den Nahrungsspezialisten *Junonia coenia* (Nymphalidae) konnten mit dem Standardsubstrat 4-Nitrophenyl-β-D-Glucose jeweils zwei β-Glucosidasen nachgewiesen werden, deren pH-Optima zwischen pH 6,4 und pH 6,7 lagen. Die beiden Generalisten *E. acraea* und *G. incorrupta* haben beide sowohl eine β-Glucosidase mit einer hohen Affinität und eine β-Glucosidase mit einer niedrigen Affinität zu 4-Nitrophenyl-β-D-Glucose. Bei dem dritten Generalisten *S. virginica* und dem Spezialisten *J. coenia* belegten Hitzeinaktivierungsexperimente, dass beide Arten jeweils eine hitzelabile und eine hitzestabile β-Glucosidase im Mitteldarm haben. Diese unterschieden sich nicht in ihrer Affinität zu 4-Nitrophenyl-β-D-Glucose und wurden bei hohen Substratkonzentrationen inhibiert.

Die Gesamt-β-Glucosidase-Aktivität der Generalisten und des Spezialisten wurde von den verschiedenen Wirtspflanzen signifikant beeinflusst. Alle drei Generalisten verringerten die β-Glucosidase-Aktivität, die mit 4-Nitrophenyl-β-D-Glucose nachgewiesen wurde, mit zunehmenden Iridoidglycosidkonzentrationen in der Pflanze. Bei den Raupen des Generalisten *G. incorrupta* auf *P. lanceolata* (hohe Iridoidglycosidkonzentrationen) korrelierte die β-Glucosidase-Aktivität signifikant negativ mit dem Larvalgewicht. Möglicherweise handelt es sich bei der Verringerung der β-Glucosidase-Aktivität um eine adaptive Reaktion auf hohe Iridoidglycosidkonzentrationen in der Nahrung. Im Gegensatz dazu erhöhte der Iridoidglycosidspezialist *J. coenia* seine β-Glucosidase-Aktivität (bestimmt mit 4-Nitrophenyl-β-D-Glucose), wenn er auf *P. lanceolata* (hohe Iridoidglycosid-konzentrationen) gehalten wurde. Das Larvalgewicht und die β-Glucosidase-Aktivität des Spezialisten *J. coenia* korrelierten hingegen nicht. Dies deutet darauf hin, dass diese β-Glucosidase des Spezialisten vermutlich keinen Effekt auf das Larvalwachstum hat. Als zweites β-Glucosidase-Substrat wurde das Iridoidglycosid Aucubin verwendet, da es in beiden iridoidglycosidhaltigen *Plantago*-Arten in unterschiedlichen Konzentrationen vorkommt. Für alle vier Lepidopteren-Arten konnte β-Glucosidase-Aktivität mit dem Substrat Aucubin nachgewiesen werden, wobei die Aktivität bei allen Raupen, die nicht auf *P. lanceolata* gehalten wurden, sehr gering war. Bei den Raupen des Generalisten *S. virginica*, die nur zwei Wochen auf *P. lanceolata* gehalten wurden, war die β-Glucosidase-Aktivität gegenüber Aucubin auf allen drei Wirtspflanzen sehr niedrig. Im Vergleich dazu war bei den Raupen des Spezialisten *J. coenia* und der beiden Generalisten *E. acraea* und *G. incorrupta*, die auf *P. lanceolata* gehalten wurden, die β-Glucosidase-Aktivität

signifikant höher als auf den übrigen Wirtspflanzen. Die Bestimmung der Michaeliskonstante für Aucubin ergab, dass sowohl die beiden Generalisten *G. incorrupta* und *E. acraea* als auch der Spezialist *J. coenia* nur eine Aucubin hydrolysierende β-Glucosidase aufwiesen. Die K_m-Werte der Aucubin hydrolysierenden β-Glucosidase der beiden Generalisten und des Spezialisten waren ähnlich und zeigten für alle drei Arten eine hohe Substrataffinität zu Aucubin an. Eine β-Glucosidase mit einer hohen Affinität zu Iridoidglycosiden erreicht schon bei niedrigen Iridoidglycosidkonzentrationen seine halbmaximale Geschwindigkeit, wodurch schon bei niedrigen Iridoidglycosidkonzentrationen große Mengen an schädlichen Aglykonen freigesetzt werden. Das Ergebnis, dass die β-Glucosidase des Spezialisten *J. coenia* eine hohe Substrataffinität zu Iridoidglycosiden hat, widerspricht der Hypothese, dass Spezialisten im Gegensatz zu Generalisten eher β-Glucosidasen mit einer geringen Affinität zu toxischen Glycosiden haben sollten, die den Spezialisten wiederum Selektionsvorteile auf den entsprechenden Wirtspflanze bringen würde.

Bei dem Generalisten *G. incorrupta* war die β-Glucosidase-Aktivität gegenüber Aucubin negativ mit dem Larvalgewicht korreliert, was vermuten lässt, dass die β-Glucosidase-Aktivität gegenüber Aucubin für die deutlich verlängerte Larvalentwicklung und das verringerte Larvalgewicht der Raupen auf *P. lanceolata* verantwortlich ist. Im Vergleich dazu entwickelten sich die Raupen auf dem iridoidglycosidfreien *T. officinale* signifikant schneller und wogen signifikant mehr. Die Raupen des Generalisten *E. acraea* und die des Spezialisten *J. coenia* wiesen im Vergleich zu *G. incorrupta* eine deutlich höhere β-Glucosidase-Aktivität gegenüber Aucubin auf. Bei dem Generalisten *E. acraea* war die β-Glucosidase-Aktivität gegenüber Aucubin positiv mit dem Larvalgewicht korreliert, während bei dem Iridoidglycosidspezialisten *J. coenia* keine Korrelation zwischen β-Glucosidase-Aktivität und Larvalgewicht vorlag. Trotz der schädlichen Iridoidglycosidaglykone, die durch die hohe β-Glucosidase-Aktivität gegenüber Aucubin freigesetzt werden, weisen eine positive bzw. nicht signifikante Korrelation zwischen β-Glucosidase-Aktivität und Larvalgewicht bei dem Generalisten *E. acraea* und dem Spezialisten *J. coenia* daraufhin, dass die Aucubin hydrolysierende β-Glucosidase bei beiden Arten wahrscheinlich keinen negativen Einfluss auf das Larvalwachstum hat.

Innerhalb der polyphagen Arctiidae hat sich die Verringerung der β-Glucosidase-Aktivität vermutlich als adaptive Reaktion auf hohe Glycosidkonzentrationen in verschiedenen Wirtspflanzen entwickelt. Allerdings weist die hohe β-Glucosidase-Aktivität gegenüber Aucubin, die bei dem Generalisten *E. acraea* positiv mit dem Larvalgewicht korreliert, ähnlich wie bei dem Spezialisten *J. coenia* eher darauf hin, dass β-Glucosidase nicht das Schlüsselenzym für die Anpassung an Iridoidglycoside darstellt. Dafür spricht auch die hohe Substrataffinität der β-Glucosidase des Iridoidglycosidspezialisten zu Aucubin. Wahrscheinlicher ist hingegen, dass beide Arten über weitere Mechanismen verfügen, durch welche freigesetzte Iridoidglycosidaglykone entgiftet oder unschädlich gemacht werden oder die Freisetzung der Aglykone verhindert wird.

1 Allgemeine Einleitung

Coevolution von Pflanzen und Insekten – war zuerst das Huhn oder das Ei?

Schon Ende der 1950er Jahre wurde in zwei richtungsweisenden Publikationen die Hypothese aufgestellt, dass Pflanzen und herbivore Insekten miteinander coevolvieren (Ehrlich & Raven, 1964, Fraenkel, 1959). Ehrlich und Raven (1964) haben diese Hypothese basierend auf einer sehr interessanten Beobachtung aufgestellt. Im Regelfall sind nah verwandte Schmetterlingstaxa mit Wirtspflanzentaxa assoziiert, die ebenfalls nah miteinander verwandt sind. Ehrlich und Raven gingen davon aus, dass verwandte Pflanzen über ein ähnliches Spektrum an Pflanzeninhaltsstoffen verfügen. Die „raison d'être" von Pflanzeninhaltsstoffen wird seit der Publikation von Fraenkel (1959) der Abwehr von herbivoren Fraßfeinden und Pathogenen zugeschrieben, wobei die meisten Pflanzeninhaltsstoffe auf Herbivoren eine fraßabschreckende Wirkung zeigen und nicht direkt letal sind. Andererseits beschrieb Fraenkel (1959) auch für verschiedene Pflanzenfamilien, dass hochspezialisierte Insekten eben die Pflanzeninhaltsstoffe dieser Pflanzenfamilien als Fraßstimulantien bzw. Lockstoffe nutzten. Genau diese Beobachtung führte Ehrlich und Raven (1964) zu der Annahme, dass Schmetterlinge und andere phytophage Insekten an bestimmte pflanzliche Sekundärstoffe spezifisch angepasst seien und mit den Pflanzen coevolvieren. Sie argumentierten, dass die Coevolution beider Gruppen durch adaptive Radiationen letztlich sowohl zu der heute beobachteten Diversität der Angiospermen als auch der phytophager Insekten geführt hat. Diese Hypothese konnte lange Zeit nicht schlüssig bewiesen werden.

Mit Hilfe von molekularbiologischen Methoden konnten diesbezüglich in den letzten Jahren neue Erkenntnisse gewonnen werden. Ward et al. konnten (2003) zeigen, dass ursprünglichere phytophage Insektentaxa eher ursprünglichere Pflanzentaxa als Wirtspflanzen nutzen. Höher entwickelte Insektentaxa sind hingegen eher mit höher entwickelten Wirtspflanzentaxa assoziiert und weisen eine größere Diversität auf. Andere Autoren konnten ebenfalls phylogenetische Assoziationen zwischen Insekten und ihren Wirtspflanzentaxa aufzeigen, was die Hypothese der Coevolution von Insekten und Pflanzen untermauert (Farrell, 1998, Farrell & Mitter, 1998, Hunt et al., 2007, Mckenna et al., 2009, Menken et al., 2009, Ødegaard et al., 2005, Weiblen et al., 2006). Die Basis für die Anpassung von phytophagen Insekten an Wirtspflanzen, die bestimmte sekundäre Pflanzeninhaltsstoffe enthalten, bildet vermutlich die Entwicklung von spezifischen Entgiftungsmechanismen. Tatsächlich konnte für verschiedene Lepidopterentaxa gezeigt werden, dass die Entwicklung von neuen

Entgiftungsenzymen die Coevolution mit den entsprechenden Wirtspflanzenfamilien ermöglicht hat (Berenbaum, 1983, Li et al., 2003, Wheat et al., 2007). Diese evolutionären Innovationen führten dann zu adaptiven Radiationen innerhalb der Lepidopterentaxa (Berenbaum, 1983, Li et al., 2003, Wheat et al., 2007). Durch die adaptive Radiation eines Insektentaxons erhöhen sich wiederum der Fraßdruck und damit der Selektionsdruck auf die Pflanzen, der nun zu einer Coevolution der Pflanzeninhaltsstoffe im Wirtspflanzentaxon und einer Weiterentwicklung der Entgiftuntsmechanismen innerhalb des Insektentaxons führt.

Iridoidglycoside und ihre Toxizität

Iridoidglycoside sind sekundäre Pflanzeninhaltsstoffe, die in über 50 verschiedenen Pflanzenfamilien innerhalb der Asteridae vorkommen (Bowers, 1991, Jensen, 1991, Jensen et al., 1975). Mittlerweile werden Iridoidglycoside als synapomorphes Merkmal der Asteriden gedeutet (Albach et al., 2001, Olmstead et al., 1993). Iridoidglycoside sind pflanzliche Glycoside, die alle ein ähnliches Grundgerüst aufweisen: Iridoidglycoside bestehen aus einem Monoterpengerüst, das über eine β-glucosidische Bindung mit einem Zuckermolekül – im Regelfall β-D-Glucose – verknüpft ist (Bowers, 1991). Verschiedene funktionelle Gruppen am Monoterpengerüst, wie beispielsweise unterschiedliche Zuckerreste, Epoxygruppen und andere, führen zu einer hohen chemischen Diversität innerhalb der Iridoidglycoside, weshalb sie auch als chemotaxonomische Merkmale verwendet werden (Jensen, 1991, Sampaio-Santos & Kaplan, 2001).

Ökologische Untersuchungen von iridoidglycosidhaltigen Pflanzen haben gezeigt, dass Iridoidglycoside der Pflanze chemischen Schutz gegen Fraßfeinde verleihen. Für nicht angepasste Insekten sind Iridoidglycoside fraßhemmend/fraßabschreckend (Bernays & De Luca, 1981, Biere et al., 2004, Puttick & Bowers, 1988). Wird die Pflanze trotz der Iridoidglycoside von Herbivoren gefressen, wirken die Iridoidglycoside zudem noch auf einer weiteren Ebene (engl. *post-digestive*) schädigend für die Herbivoren. Beispielsweise führen Iridoidglycoside bei nicht angepassten Insekten zu verringerten Wachstumsraten und einer Verlängerung des Larvalstadiums und sogar zu erhöhter Mortalität (Bowers & Puttick, 1988, Puttick & Bowers, 1988). Diese negativen Auswirkungen auf nicht angepasste Insekten werden der spezifischen Toxizität der Iridoide zugeschrieben. Im Gegensatz dazu haben sich bei verschiedenen herbivore Insekten Spezialisierungen ausgebildet, die es diesen ermöglichen, iridoidglycosidhaltige Pflanzen bevorzugt zu nutzen. Von verschiedenen Lepidopteren ist bekannt, dass Iridoidglycoside beispielsweise stimulierend auf die Eiablage

wirken (Nieminen et al., 2003, Pereyra & Bowers, 1988). Die Larven wiederum nutzen ausschließlich oligo- oder monophag iridoidglycosidhaltige Pflanzen als Wirtspflanzen, wobei die Iridoidglycoside als Fraßstimulantien dienen (Bowers, 1983, Bowers, 1984). Zudem ist bekannt, dass Iridoidglycoside von den Larven sequestriert werden (Bowers & Puttick, 1986, Gardner & Stermitz, 1988, Mead et al., 1993, Stermitz et al., 1988, Wahlberg, 2001, Willinger & Dobler, 2001) und als Fraßschutz dienen (Camara, 1997b, Dobler, 2001, Rayor & Munson, 2002, Strohmeyer et al., 1998, Theodoratus & Bowers, 1999).

Die Toxizität von Iridoidglycosiden wird einerseits auf funktionelle Gruppen am Monoterpengrundgerüst wie z. B. Epoxygruppen und des Weiteren auf die Freisetzung der hochreaktiven Aglykone zurückgeführt (Kim et al., 2000, Konno et al., 1999). Wie bei vielen anderen Pflanzentaxa mit toxischen Glycosiden spielt die Spaltung der β-glucosidischen Bindung eine wichtige Rolle. Erst durch das Enzym β-Glucosidase wird das Aglykon aus dem nicht reaktiven Iridoidglycosid freigesetzt (Bartholomaeus & Ahokas, 1995, Kim et al., 2000, Ling et al., 2003). Das Aglykon ist hochreaktiv und geht unspezifisch mit alkylierbaren Aminosäuren (Lysin, Histidin, Cystein und Methionin) kovalente Bindungen ein (Konno et al., 1999, Wink, 2006). Durch die Reaktion der Aglykone mit den nucleophilen NH_2- und SH-Gruppen der Proteine erfahren diese eine Konformationsänderung. Es kommt einerseits zur Ausfällung und auch zu einer Komplexierung der Proteine (Bartholomaeus & Ahokas, 1995, Konno et al., 1999, Wink, 2006). Proteinkomplexierung kann zu einer Verminderung der Proteinqualität und -verfügbarkeit in der Nahrung führen, was wiederum eine negative „Performance“ der Larven von nicht angepassten Insekten nach sich zieht (Felton, 1996, Felton et al., 1992).

Ist β-Glucosidase das Schlüsselenzym für die Anpassung an Iridoidglycoside?

β-Glucosidase (EC 3.2.1.21) gehört zu der Klasse der Glycosylhydrolasen und ist ein ubiquitäres Enzym, das sowohl bei den Bakterien, den Archaea und den Eukarya vorkommt (Davies & Henrissat, 1995, Henrissat & Romeu, 1995). β-Glucosidasen zeichnen sich dadurch aus, dass sie eine breite Substratspezifität besitzen und entsprechend viele verschiedene glycosidische Substanzen als Substrate nutzen können. In Pflanzen haben β-Glucosidasen verschiedene und wichtige physiologische Funktionen, wie z. B. die Bildung von Intermediaten bei der Zellwandlignifizierung, den Abbau der Zellwand bei der Samenkeimung und die Aktivierung von Phytohormonen (Esen, 1993, Verdoucq et al., 2004). Zudem spielt β-Glucosidase bei der Aktivierung von chemischen Abwehrstoffen in der

Pflanze eine wichtige Rolle (Morant et al., 2008). Tatsächlich konnte für viele verschiedene Typen von pflanzlichen glucosidischen Abwehrstoffen gezeigt werden, dass die Pflanzen zu den Abwehrstoffen auch noch eine β-Glucosidase mit entsprechender Substrataffinität mitliefern (siehe Review von Morant et al., 2008). Allgemein sind die pflanzlichen Abwehrstoffe als Glucoside biologisch inaktiv und werden in einem anderen pflanzlichen Kompartiment der Zelle gespeichert. Wird nun die Kompartimentierung der Pflanzenzelle aufgehoben, da beispielsweise ein Herbivor an der Pflanze frisst, kommen glucosidische Abwehrstoffe und β-Glucosidase in Kontakt (Morant et al., 2008). Das Enzym β-Glucosidase hydrolysiert die β-glucosidische Bindung, wobei aus den glucosidische Abwehrstoffen die Aglykone freigesetzt werden. Diese sind im Regelfall biologisch aktiv und toxisch (Yu, 1989). Pflanzliche β-Glucosidasen, die spezifisch Iridoide als Abwehrstoffe hydrolysieren, sind für Oleaceae und Apocynaceae nachgewiesen worden (Boonclarm et al., 2006, Konno et al., 1999, Mazzuca et al., 2006, Spadafora et al., 2008, Wang et al., 2009).

Auch in tierischen Organismen spielen β-Glucosidasen eine wichtige Rolle. Bei Insekten sind β-Glucosidasen hauptsächlich als Verdauungsenzyme nachgewiesen worden (Terra & Ferreira, 1994, Terra & Ferreira, 2005). β-Glucosidasen kommen im Verdauungstrakt mit verschiedenen Substraten und – je nach Wirtspflanze – auch mit pflanzlichen glycosidischen Abwehrstoffen in Kontakt, aus denen je nach Substrataffinität die giftigen Aglykone freigesetzt werden können. Aus diesem Grund wird β-Glucosidase eine wichtige Rolle bei der Anpassung von Herbivoren an toxische pflanzliche Glycoside zugesprochen (Ferreira et al., 1997). Für verschiedene herbivore Insekten konnte gezeigt werden, dass beispielsweise die Verminderung der β-Glucosidase-Aktivität eine erhöhte Toleranz von toxischen Glucosiden in der Nahrung ermöglicht (Desroches et al., 1997, Ferreira et al., 1997, Lindroth, 1988). Häufig verfügen Insekten über mehrere β-Glucosidasen, die unterschiedliche Substrataffinitäten zu verschiedenen pflanzlichen Glycosiden aufweisen (Terra & Ferreira, 1994, Terra & Ferreira, 2005). Frisst ein Insekt nun auf einer Wirtspflanze mit bestimmten toxischen Glycosiden, so kann die Freisetzung von toxischen Aglykonen dadurch verringert werden, dass gezielt die Aktivität der β-Glucosidase mit einer hohen Affinität zu dem toxischen Substrat vermindert wird (Ferreira et al., 1997). Die Beibehaltung einer hohen Enzymaktivität von anderen β-Glucosidase-Isoformen bringt den Vorteil, dass weiterhin Glucose aus anderen β-Glucosidase-Substraten freigesetzt werden kann. Somit stellt die Entwicklung von Enzymen mit geringerer Substrataffinität zu toxischen Glycosiden eine potentielle Anpassung an Wirtspflanzen dar, welche diese Glycoside enthalten.

Vergleich von β-Glucosidase bei herbivoren Generalisten und Spezialisten

Bislang konnte noch nicht gezeigt werden, welche biochemischen Anpassungen es herbivoren Insekten ermöglicht, iridoidglycosidhaltige Pflanzen als Wirtspflanzen zu nutzen. Aufgrund des oben beschriebenen Toxizitätsmechanismus von pflanzlichen Glycosiden im Allgemeinen und Iridoidglycosiden im Speziellen bietet das Enzym β-Glucosidase einen vielversprechenden Ansatzpunkt, um bei Nahrungsgeneralisten und Nahrungsspezialisten unterschiedliche Grade der Anpassung an Iridoidglycoside vergleichend zu untersuchen. Lepidopteren sind hierfür sehr geeignete Versuchsorganismen, da sich in diesem Taxon sehr unterschiedliche Stufen der Nahrungsspezialisierung entwickelt haben.

Für meine Dissertation habe ich verschiedene Schmetterlingsarten ausgewählt, um die Hypothese zu testen, dass β-Glucosidase ein potentielles Schlüsselenzym für die Anpassung an iridoidglycosidhaltige Pflanzen darstellt. In den nächsten drei Kapiteln möchte ich auf die Gemeinsamkeiten und Unterschiede eingehen, die ich bei den herbivoren Generalisten im Vergleich zu dem ausgewählten Iridoidglycosidspezialisten gefunden habe. Im ersten Kapitel meiner Dissertation untersuche ich den Einfluss von iridoidglycosidhaltigen Wirtspflanzen auf die β-Glucosidase-Aktivität des nordamerikanischen Nahrungsgeneralisten *Spilosoma virginica* (Arctiidae), wobei ich grundlegende biochemische Charakterisierungen der β-Glucosidase-Aktivität vorstelle. Im zweiten Kapitel setze ich die β-Glucosidase-Aktivität eines weiteren Nahrungsgeneralisten, der nordamerikanischen Bärenspinnerart *Grammia incorrupta* (Arctiidae), ins Verhältnis zu den Wachstumsraten der Raupen. Diese wurden entweder auf Wirtspflanzen mit oder ohne Iridoidglycosiden gehalten. Im dritten Kapitel vergleiche ich den nordamerikanischen Nahrungsgeneralisten *Estigmene acraea* (Arctiidae) mit dem nordamerikanischen Iridoidglycosidspezialisten *Junonia coenia* (Nymphalidae). Dabei berücksichtige ich die Auswirkungen, die unterschiedliche Konzentrationen von Iridoidglycosiden zweier Wirtspflanzenarten auf die β-Glucosidase-Aktivität der Larven haben. In der abschließenden Diskussion gehe ich auf die verschiedenen Ergebnisse der einzelnen Kapitel ein und diskutiere die Rolle von β-Glucosidase als Schlüsselenzym für die Anpassung an Iridoidglycoside bei herbivoren Generalisten und Spezialisten.

2 Influence of iridoid glycoside containing host plants on midgut β-glucosidase activity in the yellow woolly bear, *Spilosoma virginica* (Arctiidae)

2.1 Summary

Iridoid glycosides are secondary plant compounds that have deterrent, growth reducing or even toxic effects on non-adapted herbivorous insects. To investigate the effects of iridoid glycoside containing plants on the digestive metabolism of a generalist herbivore, larvae of *Spilosoma virginica* (Lepidoptera: Arctiidae) were reared on three plant species that differ in their secondary plant chemistry: *Taraxacum officinale* (no iridoid glycosides), *Plantago major* (low iridoid glycoside content), and *P. lanceolata* (high iridoid glycoside content). Midguts of fifth instar larvae were assayed for the activity and kinetic properties of β glucosidase using different substrates. Compared to the larvae on *T. officinale*, the β glucosidase activity of larvae feeding on *P. lanceolata* was significantly lower measured with 4-nitrophenyl-β-D-glucoside. Using the iridoid glycoside aucubin as substrate, we did not find differences in the β-glucosidase activity of the larvae reared on the three plants. Heat inactivation experiments revealed the existence of a heat-labile and a more heat-stable β-glucosidase with similar Michaelis constant for 4-nitrophenyl-β-D-glucoside. We discuss possible mechanisms leading to the observed decrease of β-glucosidase activity for larvae reared on *P. lanceolata* and its relevance for generalist herbivores in adapting to iridoid glycoside containing plant species and their use as potential host plants.

2.2 Introduction

Iridoid glycosides are secondary plant compounds that occur in over 50 plant families in many asterid clades (Albach et al., 2001, Bowers, 1991). These compounds are cyclopentanoid monoterpene-derived compounds consisting of eight-, nine- or ten-carbon skeletons with an attached monosaccharide at C-1, normally β-D-glucose (Boros & Stermitz, 1990). Like other secondary plant compounds, iridoids have deterrent effects on non-adapted insects (Bernays & De Luca, 1981, Puttick & Bowers, 1988). In addition, iridoid glycosides exert detrimental postingestive effects during larval development leading to a lower efficiency of conversion of ingested food, a lower relative growth rate, reduced larval weight, and a higher larval mortality (Bowers & Puttick, 1988, Puttick & Bowers, 1988, Stamp & Meyerhoefer, 2004). These toxic physiological effects are often dose-dependent and differ among individual iridoid glycosides depending on their chemical structure and the presence or absence of functional groups (Bernays & De Luca, 1981, Bowers & Puttick, 1988, Puttick & Bowers, 1988).

In addition to these ecological effects on herbivorous insects, the toxic mechanism of iridoid glycosides has been examined. In general, to exert toxic effects, iridoid glycosides have to be "activated" by enzymes or non-enzymatically by acid-hydrolysis. To date, only the hydrolytic β-glucosidases (EC 3.2.1.21) have been shown to convert the non-reactive iridoid glycosides into highly reactive aglycones (Kim et al., 2000, Konno et al., 1999). The resulting iridoid aglycone is a dialdehyde that is similar to the structure of glutaraldehyde in aqueous solution (Bartholomaeus & Ahokas, 1995). The mechanism for toxicity of iridoid glycosides is hypothesized to resemble that of alkylating agents such as glutaraldehyde that bind covalently to nucleophilic side chains (e.g., ε-NH_2 of Lysine) via imine formation (Bartholomaeus & Ahokas, 1995, Kim et al., 2000, Konno et al., 1997, Konno et al., 1999). Several studies show that, after hydrolysis by β-glucosidases, iridoid aglycones cross-link proteins and, depending on their chemical structure, may act as enzyme inhibitors (Bartholomaeus & Ahokas, 1995, Ling et al., 2003, Park et al., 2007).

β-Glucosidases are ubiquitous enzymes that are present in plants as well as insects (Boonclarm et al., 2006, Morant et al., 2008, Zagrobelny et al., 2004), where they are mainly found in the insect midgut or specialized tissues like defensive glands. Insect β-glucosidases act on a variety of substrates, including plant glycosides (Terra & Ferreira, 1994, Terra &

Ferreira, 2005, Yu, 1989). Based on their relative catalytic efficiency towards several substrates, insect β-glucosidases are divided into two classes (Terra & Ferreira, 2005). The β-glucosidases belonging to class A efficiently hydrolyze substrates with hydrophilic aglycones, such as disaccharides and oligosaccharides with β-1,3, β-1,4, and β-1,6 glycosidic bonds. Class B β-glucosidases include enzymes that are only active towards substrates with hydrophobic aglycones, such as alkyl-, 4-nitrophenyl-, methylumbelliferyl-glycosides as well as plant glycosides. In addition to their role in metabolizing plant glycosides and thereby providing nutritionally valuable monosaccharides for the insect, β-glucosidases play an important role in plant-insect interactions (Terra & Ferreira, 2005). According to their substrate specifity, they potentially release toxic aglycones from detrimental plant glycosides that might lead to autotoxicity. The insect β-glucosidases thus potentially influence whether herbivores might use plant glycoside containing plants as hosts or not (Ferreira et al., 1997, Yu, 1989).

To date, very little is known about the physiological mechanisms that allow insects to resist and overcome the detrimental effects of iridoid glycosides. Several hypotheses might explain the ability of generalist and specialist insects to tolerate iridoid glycosides in their host plants. (1) Herbivorous insects could be capable of producing enzymes that either detoxify the iridoid glycosides or their aglycones. (2) Insects may overcome the toxic effects of the activated iridoids by secreting free amino acids into the midgut that act as buffers (Konno et al., 1997, Konno et al., 2001). Konno et al. (1997, 2001) showed that some lepidopteran larvae actively secrete free glycine into the midgut which protects them against the strong protein-denaturing activity of oleuropein, a phenolic iridoid in their host plant, *Ligustrum obtusifolium* (Oleaceae). (3) Insects could possess specific broad-specifity β-glucosyltransferases (EC 2.4.1.) reconverting the released aglycones to non-toxic glycosides (Ahmad & Hopkins, 1993). (4) To avoid the effects of toxic plant β-glycosides, insects could down-regulate the enzyme most active towards the glycosides. Some studies showed that, for several coleopteran and lepidopteran species, tolerance of toxic plant glycosides in their food was achieved by a reduction of β-glucosidase activity in the gut (Desroches et al., 1997, Ferreira et al., 1997, Lindroth, 1988). (5) Low enzymatic substrate specificity towards the toxic plant compounds could lead to tolerance, as the toxic compound would be poorly hydrolyzed or unhydrolyzed after consumption (Ferreira et al., 1997, Marana et al., 2000).

The aim of the present study was to investigate the underlying physiological mechanisms that could lead generalist herbivorous insects to tolerating iridoid glycosides in their diet. For that, we chose the generalist caterpillar *Spilosoma virginica* (Lepidoptera: Arctiidae). This generalist species is known to feed on more than 100 different plant species including taxa that contain iridoid glycosides (Dethier, 1988, Tietz, 1972). *S. virginica* larvae do feed on iridoid glycoside containing plants, but were shown to not sequester iridoid glycosides (Bowers & Stamp, 1997). To test whether midgut β-glucosidase activity in *S. virginica* larvae was influenced by host plant chemistry, we chose three natural host of *S. virginica* that differ in their iridoid glycoside content and composition. We chose a plant species without iridoid glycosides as control in our experiment, a plant species with a low amount of iridoid glycosides and a plant species with a high amount of iridoid glycosides: *Taraxacum officinale* (Asteraceae) (no iridoids), *Plantago major* (Plantaginaceae) (low iridoids) and *Plantago lanceolata* (high iridoids). Although *T. officinale* does not contain iridoid glycosides, it does contain other bitter compounds belonging to the group of sesquiterpene lactones, as well as high amounts of phenolic compounds (Schütz et al., 2006, Schütz et al., 2005, Williams et al., 1996). *T. officinale* is one of the preferred host plants of *S. virginica* larvae, as is *P. major* (Dethier, 1988), which contains low amounts of iridoid glycosides. The major iridoid glycoside in *P. major* is aucubin which occurs in amounts ranging from 0.07 to 1.0% of the plant dry weight (Barton & Bowers, 2006, Rønsted et al., 2003, Willinger & Dobler, 2001). In comparison to *P. major*, *P. lanceolata* contains high amounts of iridoid glycosides, primarily aucubin and catalpol (Barton & Bowers, 2006, Rønsted et al., 2003, Rønsted et al., 2000, Willinger & Dobler, 2001). The amount of the iridoid glycosides and the catalpol:aucubin ratio of *P. lanceolata* differs among populations and genotypes, and also depends on the position of the leaf in the rosette (new vs. old leaf), the plant age and the neighboring plants (Barton & Bowers, 2006, Bowers & Stamp, 1993, Fuchs & Bowers, 2004, Marak et al., 2000, Wink, 2006). Leaves of *P. lanceolata* sampled in Germany and the Netherlands have a total iridoid glycoside concentration between 1-2 % of the plant dry weight with a catalpol:aucubin ratio usually lying between 0.5 to 0.7 (Fontana et al., 2009, Marak et al., 2000, Willinger & Dobler, 2001, Wurst & Van Der Putten, 2007). For addressing the ultimate question of how the polyphagous species is able to feed on iridoid glycoside containing plants on a biochemical level, we examined as well the biochemical of nature β-glucosidase by performing kinetic studies and examining the thermal stability of the midgut β-glucosidases in *S. virginica* larvae.

Overall, we designed this research to (1) determine midgut β-glucosidase activity of *S. virginica* larvae in relationship to plants that differ in their secondary plant chemistry; (2) determine the substrate affinity of *S. virginica* β-glucosidase towards the substrates 4-nitrophenyl-β-D-glucoside and aucubin; (3) determine thermal stability of β-glucosidase isoforms in *S. virginica* by heat inactivation experiments; (4) address the underlying physiological mechanisms that could lead generalist insect herbivores to tolerating iridoid glycosides in their diet.

2.3 Materials and Methods

Insect rearing

Larvae of *S. virginica* were from a laboratory culture from Deane Bowers (University of Colorado, Ecology and Evolutionary Biology, Boulder, CO 80309, USA) and maintained at the University of Hamburg in the summer 2004. Larvae were reared in plastic containers at 22 °C and a 16:8 h light:dark photoperiod until their fifth instar. Larvae were fed with fresh leaves of *T. officinale* through their fourth instar. After molting to the fifth instar, larvae were put on a different diet for approximately two weeks prior to dissection. The larvae continued feeding on *T. officinale* or were fed exclusively with fresh leaves of *P. major* or *P. lanceolata*. Host plants were collected every week from local populations, washed and stored in a refrigerator at 4° C until use.

Enzyme preparation

Caterpillars were immobilized in the refrigerator at 4 °C for 10 minutes before dissection. Guts were dissected in cold 0.1 M sodium acetate buffer, pH 5.0. Midguts were isolated and rinsed to remove the midgut contents. The gut sections were then homogenized in cold 0.1 M sodium acetate buffer pH 5.0 with a pestle. Then, all samples were centrifuged at 2 °C for 45 minutes at 10.000 g and the resulting supernatants were collected and used as a source of enzymes. Due to idiosyncrasies of a previous experimental design, supernatants were thawed and frozen twice before storing the aliquots at -80 °C. The enzyme preparations could be stored for at least five years at -80 °C without any noticeable change in the activity of the enzymes assayed.

Protein determination and enzyme assays

Protein concentration of each sample was determined with Bradford reagent (Sigma Aldrich, Munich, Germany) by measuring the absorbance at 595 nm and comparing it to a standard curve of BSA. To determine optimal reaction conditions, the pH optimum for the β-glucosidase was identified using 0.1 M citrate sodium phosphate buffer by varying the pH from 6.0 to 7.4 in steps of pH 0.2. The substrate 4-nitrophenyl-β-D-glucoside (NPβGlc) from which the chromogenic 4-nitrophenolate is cleaved off by β-glucosidases, was used to determine β-glucosidase activity. All enzyme assays were performed at 30 °C in 100 mM citrate sodium phosphate buffer at pH 6.4 with 5.7 mM NPβGlc as substrate. Controls without enzyme and controls without substrate were always included. The enzymatic reaction was stopped by adding ice cold 0.5 M Na_2CO_3 solution (Selmar et al., 1987). The absorbance of 4-nitrophenolate was measured at 400 nm (UV-Spectrophotometer Biochrom 2100 Pro) and compared to a standard curve to determine β-glucosidase activity (Hösel & Barz, 1975). The β-glucosidase activity towards 5.0 mM aucubin was determined under the same reaction conditions as described above by quantifying the release of glucose from aucubin which is equimolar to the release of the aglycone aucubigenin. Controls without substrate were included to measure the glucose/β-NAD/ATP background within the enzyme homogenate and controls without enzyme were included to test for aucubin auto-hydrolysis. The reaction was stopped by heat inactivating the enzymes for 5 minutes at 100 °C. The amount of released glucose was determined with hexokinase and glucose-phosphate-6-dehydrogenase (Ogawa et al., 2001) with slight modifications of the protocol proposed by Ogawa et al. (2001). To test under which conditions the β-glucosidase followed initial velocity, incubations were carried out for at least eight different periods of time (0 – 130 min) and twelve different protein concentrations (0 - 25 µg). In the experiments, the β-glucosidase assays were performed under conditions such that the β-glucosidase activity was proportional to protein concentration and to time and followed initial velocity. One unit of enzyme (U) is defined as the amount of enzyme that hydrolyzes one µmol of substrate per minute at 30 °C.

Kinetic properties of β-glucosidase activity

To determine the effect of different substrate concentrations of NPβGlc on the β-glucosidase activity (ranging from 0.4 mM to 32 mM), midgut extracts from larvae reared on *T. officinale* were tested (n = 3). Controls were included for every substrate concentration used. Enzymatic activity following a steady state enzyme kinetic can be described by the Michaelis-Menten equation (1) (Copeland, 2000).

$$V = \frac{V_{\max} * C}{K_m + C} \qquad (1)$$

Here, V is the reaction velocity of the enzyme, V_{max} is the maximum reaction velocity of the enzyme, C is the substrate concentration used and K_m is the substrate concentration that provides a reaction velocity that is half of the maximal velocity obtained under saturating substrate conditions, named Michaelis constant (Copeland, 2000).

At higher substrate concentrations, substrate inhibition can cause nonhyperbolic behavior of the enzymatic activity. Here, a second molecule of substrate can bind to the enzyme-substrate complex to form an inactive ternary complex, substrate-enzyme-substrate complex. Substrate inhibition can be described by equation (2).

$$V = \frac{V_{\max} * C}{K_m + C + \frac{C^2}{Ki}} \qquad (2)$$

The term K_i represents the dissociation constant for the inhibitory substrate-enzyme-substrate ternary complex. The substrate inhibition model was further modified to include two isoenzymes with differing Michaelis-Menten constants, where one enzyme should be inhibited at higher substrate concentrations while the kinetic of the other followed a simple Michaelis-Menten behavior as shown in equation (3).

$$V = \frac{V_{\max 1} * C}{K_{m1} + C + \frac{C^2}{K_{i1}}} + \frac{V_{\max 2} * C}{K_{m2} + C} \qquad (3)$$

The experimental data were fitted to equation (1), (2) and (3) and kinetic parameters were calculated with JMP 7.1 performing an iterative non-linear regression analysis.

Heat inactivation experiments

To calculate thermal inactivation constants of β-glucosidase activity, midgut extracts of *S. virginica* larvae reared on *T. officinale* (n = 3) were incubated in 0.1 M citrate sodium phosphate buffer at pH 6.4 at 45 °C for different time intervals (0 to 150 minutes). Then, aliquots were removed and the residual β-glucosidase activity was determined at 30 °C as described above. Two exponential models that describe the inactivation kinetics for one or two enzyme isoforms differing in heat stability were tested using iterative non-linear regression analysis (Motulsky & Christopoulos, 2004).

One phase decay: $Y = (Y_0 - a) * \exp^{(-K * X)} + a$ (4)

Two phase decay: $Y = a + (Y_0 - a) * \text{Percent}_{Fast} * 0.01 * \exp^{(-K_{Fast} * X)} +$
$(Y_0 - a) * (100 - \text{Percent}_{Fast}) * 0.01 * \exp^{(-K_{Slow} * X)}$ (5)

with Y_0 being the initial activity measured at t = 0 [min], a being the residual activity at t = 120 [min] and K being the heat inactivation constant.

The half lives for the heat-labile and the heat-stable β-glucosidases were calculated as follows:

$$\text{Half life} = \frac{-\ln(\frac{1}{2})}{K}$$

The experimental data were fitted to equation (4) and (5) and parameters for heat inactivation were calculated with JMP 7.1 performing an iterative non-linear regression analysis.

Statistical analysis

For the feeding experiments with different host plants, we used a balanced experimental design. Data were tested for homogeneity of variance (Bartlett test and Levene test) and normal distribution (Shapiro Wilk test). To compare sample sizes with $k > 2$, single-factorial non-parametric ANOVAs (Kruskal-Wallis test) were performed and followed by the Nemenyi post-hoc test. To compare the fit of the kinetic as well as the heat inactivation models, we performed an F-test as proposed by Motulsky and Christopoulos (2004). As a null hypothesis, the simpler model including fewer parameters is assumed to be correct. The F-test to compare the model fit between nested models is calculated as follows, implying the sum of squares and the degrees of freedom from the computed curve statistics that you get after having performed the iterative non-linear regression analysis of the models in chapter *2.4* or *2.5* (Motulsky & Christopoulos, 2004):

$$F = \frac{(SS_1 - SS_2)/(DF_1 - DF_2)}{SS_2/DF_2}$$

An F-ratio near 1.0 means that the simpler model is correct, while a much greater F-ratio with a P-value lower than 0.05 concludes that the more complicated model fits significantly better than the simple model (Motulsky & Christopoulos, 2004). All statistical tests were performed with the statistical software JMP 7.1 (SAS) and Excel (Microsoft).

2.4 Results

Kinetic properties of β-glucosidase activity

For all tested individuals (n = 3), the kinetic of β-glucosidase in the midgut raw extract followed a Michaelis-Menten behavior when using the synthetic substrate NPβGlc in a range of 0.4 mM to 5.6 mM. At substrate concentrations higher than 5.6 mM, the Eadie-Hofstee plot clearly revealed substrate inhibition (Figure 2.1). Comparing the simple Michaelis-Menten model (see Materials and Methods, equation (1)) with the substrate inhibition model (2) for all three measured individuals, the simple Michaelis-Menten kinetic (1) was rejected for all individuals tested in favor of the Michaelis-Menten model accounting for substrate inhibition ($p < 0.001$, see Table 2.1). When comparing the substrate inhibition model with one Michaelis constant (4) with the more complex model (3) for substrate inhibition including two different Michaelis constants, the F-test yielded non-significant results. Kinetic parameters of *S. virginica* midgut β-glucosidases are given in Table 2.1.

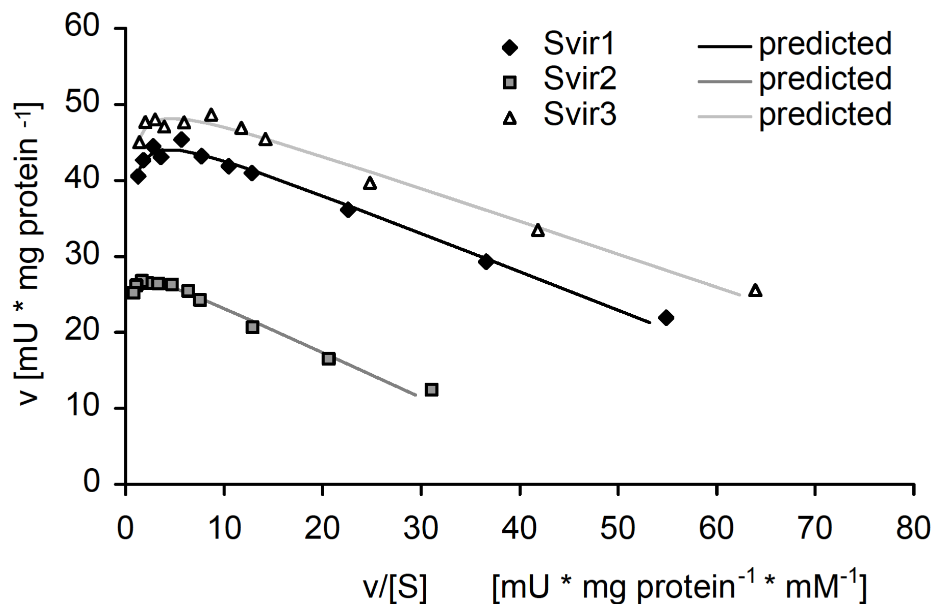

Figure 2.1 Eadie-Hofstee plot which shows the β-glucosidase activity of three individual *S. virginica* larvae (black diamonds, grey squares and white triangles) that was determined with at least eleven different concentrations of the substrate NPβGlc. The β-glucosidase activity v was plotted against the β-glucosidase activity per substrate concentration v/[S]. Each point represents the means of two independent β-glucosidase measurements that was done for each substrate concentration. The curves are theoretical predictions, and were calculated using the kinetic parameters that were estimated by iterative nonlinear regression based on the Michaelis-Menten model accounting for substrate inhibition (2).

Table 2.1 The effect of different concentrations of the substrate 4-nitrophenyl-β-D-glucoside on the β-glucosidase activity of three individual *Spilosoma virginica* larvae reared on *Taraxacum officinale* was determined. Kinetic parameters (means ± S. E) were determined by iterative nonlinear regression based on the Michaelis-Menten-model (1) or the Michaelis-Menten-model accounting for substrate inhibition (2) with K_m being the Michaelis-Menten constant, V_{max} being the maximum reaction velocity of the β-glucosidase, and Ki being the dissociation constant for the inhibitory substrate-enzyme-substrate ternary complex. P-values < 0.05 indicate that the more complex model (2) fits significantly better than the simpler model (1).

No.	Host plant	K_m [mM]	V_{max} [mU * mg protein^{-1}]	Substrate inhibition K_i [mM]	model	F-test	P-value
1	*T. officinale*	0.51 ± 0.03	48.59 ± 0.78	193.68 ± 35.84	(1) vs. (2)	$F_{1, 8} = 36.7$	$P < 0.001$
2	*T. officinale*	0.60 ± 0.03	29.35 ± 0.41	233.17 ± 42.89	(1) vs. (2)	$F_{1, 8} = 35.6$	$P < 0.001$
3	*T. officinale*	0.44 ± 0.03	52.29 ± 0.78	240.57 ± 51.19	(1) vs. (2)	$F_{1, 8} = 26.4$	$P < 0.001$

The β-glucosidase activity of the three individuals was determined at pH 6.4 in 0.1 M citrate-sodium phosphate buffer with two replicates per substrate concentration. All parameters were calculated based on the determined β-glucosidase activity for eleven substrate concentrations and represent the parameter estimate and the corresponding standard error.

Heat inactivation experiments

The inactivation curves showed an initial straight line with a steep slope (heat-labile enzyme activity) and a final line with a shallow slope (heat-stable enzyme activity) (Figure 2.2). Comparing the fit of model (4) and (5) to the data, the model of one phase exponential decay (corresponding to one β-glucosidase) is rejected in favor of the two phase decay model that describes the exponential decay velocity of two enzymatic isoforms due to differing heat stability. Model (5) provided a better fit for all three individuals tested (Table 2.2). Between 35% and 40% of the entire β-glucosidase activity measured with NPβGlc could be attributed to a more heat-labile isoform, whereas 60 to 65% correspond to a β-glucosidase with higher heat stability with the corresponding heat inactivation constants that are given in Table 2.2.

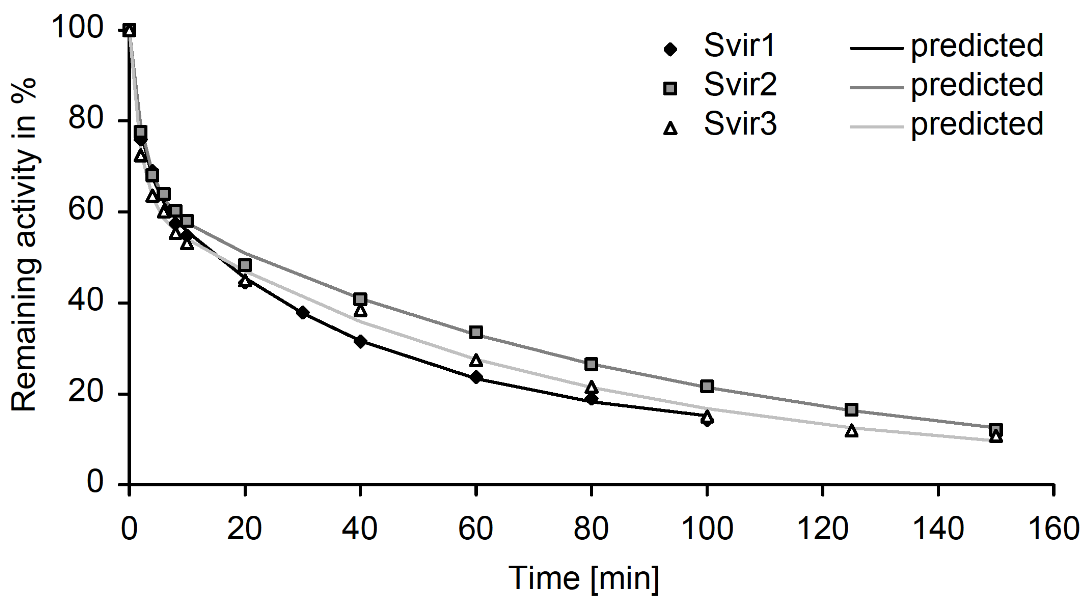

Figure 2.2 The thermal inactivation of midgut β-glucosidases at 45°C of three individual *Spilosoma virginica* larvae (black diamonds, grey squares and white triangles) that were reared on *Taraxacum officinale* is shown. After at least twelve increasing time frames in which the β-glucosidase had been incubated at 45°C, the residual β-glucosidase activity was determined with 5.7 mM NPβGlc at pH 6.4 in 0.1 M citrate-sodium phosphate buffer. The points are the mean of two replicates that were measured per time frame for each individual, whereas the curves are theoretical predictions based on the estimated parameters presented in Table 2.2. These parameters were estimated by using iterative nonlinear regression based on two-phase decay model (5) which implies the presence of a heat-labile and a heat-stable β-glucosidase in the larval midgut.

Table 2.2 Heat inactivation constants of *Spilosoma virginica* β-glucosidases were determined for three different larvae reared on *Taraxacum officinale*. Based on equation (5), which describes the two phase decay of the β-glucosidase inactivation, the heat inactivation parameters were determined by iterative nonlinear regression and represent the estimated mean ± standard error, with a being the residual β-glucosidase activity [U mg protein^{-1}] where the inactivation curve plateaus at some value (zero or other than zero), $Percent_{Fast}$ representing the percentage of the heat-labile β-glucosidase fraction in the midgut, K_{Fast} being the heat inactivation constant for the heat-labile, and K_{Slow} being the heat inactivation constant for the heat-stable β-glucosidase. The half lives of the heat-labile and the heat-stable β-glucosidase in the midgut were calculated based on the inactivation constants K_{Fast} and K_{Slow} respectively (-ln (0.5)/K). P-values < 0.05 indicate that the more complex two-phase decay model (5) fits significantly better than the simpler model for one-phase decay (4).

Parameter	**Svir_1**	**Svir_2**	**Svir_3**
Y0 =100 %	99.7 ± 1.6 %	99.6 ± 1.2 %	99.8 ± 1.6 %
Plateau a in %	11.3 ± 2.6	0.3 ± 4.2	3.4 ± 3.3
$Percent_{Fast}$ in [%]	34.1 ± 2.3	36.8 ± 1.4	39.5 ± 1.7
K_{Fast} [min^{-1}]	0.58 ± 0.12	0.38 ± 0.04	0.53 ± 0.08
K_{Slow} [min^{-1}]	0.027 ± 0.004	0.011 ± 0.002	0.015 ± 0.002
Half $life_{Fast}$ [min] = $-ln(0,5)/K_{Fast}$	1.2 ± 0.2	1.8 ± 0.2	1.3 ± 0.2
Half $life_{Slow}$ [min] = $-ln(0,5)/K_{Slow}$	26.1 ± 3.5	63.4 ± 8.9	47.0 ± 6.7
F-Test	$F_{2, 8} = 27.7$	$F_{2, 8} = 222.7$	$F_{2, 8} = 139.0$
P-value	P < 0.001	P < 0.001	P < 0.001

After at least twelve increasing time frames in which the β-glucosidase had been incubated at 45°C, the residual β-glucosidase activity was determined with 5.7 mM NPβGlc at pH 6.4 in 0.1 M citrate-sodium phosphate buffer.

Enzyme assays

When testing for the optimal reaction conditions of *S. virginica* β-glucosidase raw extracts, a maximum of β-glucosidase activity could be measured at pH 6.4 using 100 mM citrate sodium phosphate buffer. The activity profile of β-glucosidase was bell-shaped. About 95% of maximum β-glucosidase activity was retained between pH 6.0 up to pH 6.8. At pH 7.4, approximately 85% of the maximum β-glucosidase activity could be measured still. The β-glucosidase activity increased linearly with time (up to 130 min) in 100 mM citrate sodium phosphate buffer, pH 6.4 and with increasing protein concentration (up to the protein concentration used in our experimental enzyme assays).

First, we tested whether the amount of protein per midgut homogenate differed when larvae were reared on different host plants and we found that the protein concentrations of the midgut homogenates did not differ significantly (*Kruskal-Wallis test*, $H_{5,5,5} = 1.140$, $p > 0.05$). However, the specific β-glucosidase activity based on the enzymatic activity per mg protein differed significantly when the larvae were reared on different plants (*Kruskal-Wallis test*, $H_{5,5,5} = 10.22$, $p < 0.001$; Figure 2.3).

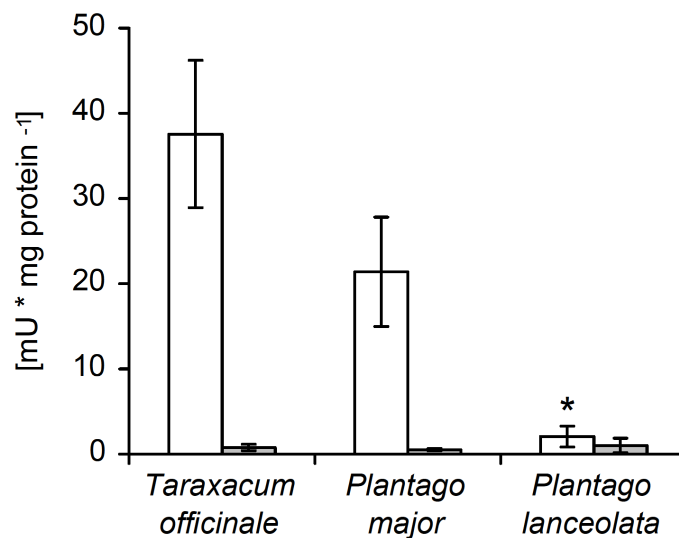

Figure 2.3 Specific β-glucosidase activity (means + S.E.) of *Spilosoma virginica* 5th instar larvae reared for two weeks exclusively on either of the following host plants, n = 5 on *Taraxacum officinale* (without iridoid glycosides), n = 5 on *Plantago major* (low iridoid glycoside content) and n = 5 on *Plantago lanceolata* (high iridoid glycoside content). For each larvae, the midgut β-glucosidase activity was determined using two substrates, either 5.7 mM NPβGlc (white bars) or 5 mM aucubin (grey bars) respectively. The asterisk (* $p < 0.025$) indicates that the β-glucosidase activity of the larvae reared on *P. lanceolata* differs significantly in comparison to the activity of the larvae reared on *T. officinale*.

While the specific β-glucosidase activity of larvae reared on *P. major* did not differ significantly from the larvae on *T. officinale*, the specific β-glucosidase activity of larvae on *P. lanceolata* was significantly lower compared to the larvae reared on *T. officinale* (*Nemenyi test*, $q_{\infty,\ 3}$= 4.400, $p < 0.025$). Using the iridoid glycoside aucubin as substrate revealed that the specific midgut β-glucosidase activity was very low. Midgut β-glucosidase activity towards aucubin did not differ significantly when the larvae were reared on the three different host plants (*Kruskal-Wallis test*, $H_{5,5,4} = 1.286$, $p > 0.10$; Figure 2.3), although the mean of the β-glucosidase activity of larvae reared on *P. lanceolata* was somewhat higher than in larvae reared on *P. major* or on *T. officinale*.

2.5 Discussion

In this study we wanted to examine the effect of host plants that differ in their secondary chemistry on the midgut β-glucosidase activity of the generalist arctiid herbivore *S. virginica*. We used the β-glucosidase standard substrate 4-nitrophenyl-β-D-glucose (NPβGlc) for class B β-glucosidases as proposed by Terra and Ferreira (2005) which are the β-glucosidases that are active towards substrates with hydrophobic aglycones such as 4-nitrophenyl-glycosides. Thereby, we assumed to detect all class B β-glucosidases which include β-glucosidases that are active against plant glycosides.

First, we examined the midgut β-glucosidase activity of *S. virginica* larvae using different substrate concentrations. For lower substrate concentrations ranging from 0.4 - 5.6 mM, we found that β-glucosidase kinetics followed a simple Michaelis-Menten behavior. However at higher substrate concentrations up to 32 mM, substrate inhibition could be observed when the activity was plotted as a function of activity per substrate concentration in an Eadie-Hofstee plot. The substrate inhibition could be described satisfactorily with the Michaelis-Menten equation accounting for substrate inhibition (2) by introducing the dissociation constant K_i into the equation which accounts for second substrate molecules binding to the enzyme-substrate complex at higher substrate concentrations. This phenomenon has been shown for other insect glycosidases (Azevedo et al., 2003, Genta et al., 2007). However in some cases, the observed pattern of substrate inhibition in β-glucosidases is an artifact. In these cases, it results from transglycosylation caused by the reaction of enzyme products with substrate molecules (Cristofoletti et al., 2003, Ferreira et al., 2001). Calculating the Michaelis constants based on equation (2), Michaelis constants were similar for the three measured individuals,

and lie in a typical range of lepidopteran Michaelis constants for NPβGlc (Azevedo et al., 2003, Marana et al., 2000, Pratviel-Sosa et al., 1987, Santos & Terra, 1985). The slight differences of the individual Michaelis constants, as well as the larger differences between the dissociation constants K_i of the three analyzed individuals, could be due to genetic causes, i.e. that the larvae may differ in their β-glucosidase alleles. Alternatively, the differences might be due to experimental conditions, i.e. the fact that we analyzed unpurified enzyme homogenates containing two β-glucosidases as suggested by the heat inactivation experiments.

Heat inactivation experiments were performed with larvae reared on *T. officinale* because of their relatively high enzymatic activity. At 45 °C, β-glucosidase activity declined exponentially in a biphasic curve over time with a very low residual activity after 120 - 150 minutes of heat treatment. The loss of enzymatic activity could be described satisfactorily with an exponential model that consists of both a fast and a slow exponential decline of enzymatic activity with differing inactivation rate constants. The results of the heat inactivation experiment were as well rather consistent for all three measured individuals, although the half lives of the heat stable β-glucosidase were between approximately 30 and 60 minutes. The results of the heat inactivation experiments suggest that *S. virginica* larvae feeding on *T. officinale* have at least two midgut β-glucosidases that differ in heat stability, where the heat-labile β-glucosidase accounts for 35 to 40% of the total β-glucosidase activity. Compared to other insect species in which β-glucosidases retain their activity at 45 °C without significant losses (Morgan, 1975, Pratviel-Sosa et al., 1987, Santos & Terra, 1985), β-glucosidases of *S. virginica* seem to be more heat-labile. In comparison to other lepidopteran β-glucosidases, *S. virginica* β-glucosidases are typical insect β-glucosidases and share characteristics like their pH-optimum and their K_m value for NPβGlc with β-glucosidases of other lepidopteran species. In our view, although unusual, it pays to determine the physiological heat inactivation and Michaelis constants for at least three different individuals to get an idea of individual differences that may be based on different alleles occurring in a population and thus may help to better understand the variability underlying the potential for adaptations to host plant chemistry.

In our feeding experiment, we reared *S. virginica* fifth instar larvae for two weeks on the three host plants species *T. officinale, P. major* and *P. lanceolata* that differed in their iridoid glycoside content. Then, we determined their midgut β-glucosidase activity with the substrates NPβGlc and the iridoid glycoside aucubin, which is present in two of our used host

plants. Based on this experimental approach, we found significant differences in midgut β-glucosidase activity between the larvae reared on different plant species. The highest mean of β-glucosidase activity for NPβGlc was observed in larvae reared on *T. officinale* that does not contain iridoid glycosides or other toxic plant glycosides. For the larvae on *P. major*, the mean β-glucosidase activity for NPβGlc was slightly lower then on *T. officinale*, yet the difference was not significant. However when the larvae in our experiment were reared on *P. lanceolata*, we found a highly significant difference in β-glucosidase activity compared to that of the larvae reared on *T. officinale*. In the larvae reared on *P. lanceolata*, the plant with the highest iridoid glycoside content in our experiment, the total midgut β-glucosidase activity determined with NPβGlc was significantly reduced compared to the β-glucosidase activity of the larvae on *T. officinale*, the plant without iridoid glycosides. For all larvae reared on *T. officinale* and *P. major*, we observed a high variance in the total β-glucosidase activity, whereas the variance of the total β-glucosidase activity of the larva on *P. lanceolata* was very low. Taking a closer look at the variances in β-glucosidase activity of larvae on different host plant is important because it shows that individuals normally show a high variability in β-glucosidase activity when feeding on less detrimental plants. However, feeding on a plant with high concentrations of detrimental glycosides, like *P. lanceolata,* leads not only to a reduction of the total β-glucosidase activity in all individuals, but also a reduction in variability of total β-glucosidase activity between different individuals. Thus, we conclude that feeding on *P. lanceolata* has a strong and significant influence on the total class B midgut β-glucosidase activity in the larvae. The reduction of the total β-glucosidase activity seems to be the only possible and obligate reaction for all *S. virginica* larvae to continue feeding on *P. lanceolata*.

Apart from NPβGlc, we used the iridoid glycoside aucubin as a β-glucosidase substrate which occurs in both plantain species in different concentrations. However, the β-glucosidase activity against aucubin was very low and did not differ between the host plants. Bowers and Stamp (1997) found low levels of iridoid glycosides in the frass from *S. virginica* larvae reared on *P. lanceolata*, which is in agreement with our results that aucubin, one of the iridoid glycosides in *P. lanceolata*, is hydrolyzed, but with a very low activity and thus intact aucubin must be excreted with the frass. The arctiids are supposed to have broadened their host plant range to include iridoid glycoside containing plant species rather recently (Conner, 2008), so it may not be surprising that a reduced substrate affinity towards these secondary compounds has not yet evolved.

To better understand the pattern of β-glucosidase activity in this species, it is important to consider the feeding ecology of *S. virginica* larvae. The larvae are typical generalist herbivores that regularly switch host plants in their natural habitat (Dethier, 1988, Tietz, 1972) and thus encounter a variety of secondary plant compounds in their diet with which they have to deal. Several different hypotheses have been proposed to explain the significance and benefits of dietary generalism in herbivores. One of these hypotheses is the "detoxification limitation hypothesis" (Freeland & Janzen, 1974, Marsh et al., 2006, Singer et al., 2002), which proposes that switching host plants helps to avoid the ingestion of toxic doses of secondary metabolites. In our no-choice trials, we reared *S. virginica* larvae for two weeks on three different host plant species, with no alternate food available. Thus, it was not possible for the larvae "to dilute high toxin concentrations". According to the detoxification limitation hypothesis, feeding exclusively on one host plant species could lead to the intake of toxic doses of secondary plant compounds in generalists for which they would not have sufficient detoxification capacity. Analyzing the β-glucosidase activity in relation to the host plants and their secondary compounds, we can deduce a specific adaptive pattern of β-glucosidase activity which is negatively correlated with the increasing iridoid glycoside concentrations of the host plants. On the plant with the highest amounts of iridoid glycosides, *P. lanceolata*, we found the lowest β-glucosidase activity in the larvae. We presume that the decrease of the total β-glucosidase activity measured with NPβGlc could be an unspecific reaction to toxic glycosides as the larvae did not show substantial β-glucosidase activity against aucubin on either of the host plants. So, the results of our feeding experiment are partially consistent with the "detoxification limitation hypothesis". During our no-choice feeding experiment, the *S. virginica* larvae on *P. lanceolata* could not control the intake of noxious plant compounds by host plant switching. The larvae responded with the total decrease of their midgut class B β-glucosidase activity which thus should minimize the probability that detrimental metabolites would be released from the iridoid glycosides.

In other insect species, a similar kind of adaptive β-glucosidase regulation in relation to toxic plant glycosides in the diet has been observed (Desroches et al., 1997, Ferreira et al., 1997, Lindroth, 1988, Mainguet et al., 2000). For example, when the sugarcane borer *Diatraea saccharalis* (Pyralidae), a generalist lepidopteran, feeds on an artificial diet containing the cyanogenic glycoside amygdalin, the activity of its three midgut β-glucosidases follows a specific induction/ reduction pattern so that the release of toxic metabolites is reduced and the larvae can feed without a significant impairment on the larval development or a higher

mortality (Ferreira et al., 1997). However in our experiment, β-glucosidase activity against aucubin was low in larvae reared on all plants and we did not find a reduction of the aucubin hydrolyzing β-glucosidase related to the host plants used. Nevertheless, a reduction of the class B midgut β-glucosidase activity might be highly useful when feeding on *P. lanceolata* as aucubin is just one of several iridoid glycosides and other possibly detrimental glycosides (Beara et al., 2008, Gálvez et al., 2005). Moreover, a strong reduction of β-glucosidase in response to plants with high plant glycoside concentrations might be useful as an unspecific adaptive response to protect the generalist from detrimental postingestive effects. The reduction of β-glucosidase activity would minimize the risk of mortality due to the reduced formation of toxic aglycones during digestion. So, the regulation of β-glucosidase activity would help the larvae to survive feeding on plants with toxic glycosides by just paying the cost of a lower monosaccharide intake due to the decrease of β-glucosidase activity. So far, to our knowledge there are no studies that have examined feedback mechanisms of insect β-glucosidase gene expression and β-glucosidase secretion in relationship to diet and/or host plants and their secondary plant chemistry. However it is possible that at higher glycoside concentrations in the diet, certain feedback mechanisms exist that lead to an unspecific adaptive reduction in β-glucosidase activity in generalists even if the species' β-glucosidase does not have the specific substrate affinity to hydrolyze the detrimental glycoside in the consumed host plant. An unspecific reduction of β-glucosidase in response to plants with high concentrations of detrimental glycosides might be helpful to circumvent the potentially toxic release of detrimental aglycones without selecting for biochemical adaptation towards specific plant compounds.

To our knowledge, this is the first time that the β-glucosidase activity of an arctiid generalist has been analyzed in relationship to host plants with iridoid glycosides. Based on the observed detrimental postingestive effects during larval development leading to a lower efficiency of conversion of ingested food, a lower relative growth rate, reduced larval weight, and a higher larval mortality (Bowers & Puttick, 1988, Puttick & Bowers, 1988, Stamp & Meyerhoefer, 2004), future experiments should relate larval performance on these host plant species to the β-glucosidase activity. This plant-insect system provides valuable insights into ways of tolerating detrimental host plant chemistry without further selecting for specific adaptations on a biochemical base in the studied enzyme.

3 The interplay between toxin-releasing β-glucosidase and plant iridoid glycosides impairs larval development in a generalist caterpillar (*Grammia incorrupta*. Arctiidae)

3.1 Summary

Feeding on iridoid glycoside containing plants by non-adapted herbivorous insects can be detrimental because iridoids are deterrent and have growth reducing or even toxic effects. Thus far, the underlying mechanisms of adaptation or tolerance which enable insects to feed on iridoid glycoside containing plants are not known. For that we investigated larval performance and the digestive metabolism of the generalist herbivore, *Grammia incorrupta* (Lepidoptera: Arctiidae). The caterpillars are known to regularly switch host plants, possibly to minimize the ingestion of harmful plant secondary metabolites. We performed a no-choice feeding experiment in which the larvae were reared exclusively on one of two plant species that differ in secondary plant chemistry. We used *Taraxacum officinale* (without iridoids), and *Plantago lanceolata* (with iridoid glycosides). The development of caterpillars reared on *P. lanceolata* was significantly prolonged compared to that on *T. officinale*. Midgut homogenates of last instar larvae were then assayed for activity and kinetic properties of β-glucosidases. In larvae feeding on *P. lanceolata*, the specific β-glucosidase towards 4-nitrophenyl-β-D-glucoside (NPβGlc) was significantly reduced. Additionally, we found an aucubin hydrolyzing β-glucosidase in the larvae reared on *P. lanceolata* but none in the larvae on *T. officinale*. The β-glucosidase activity towards NPβGlc and aucubin were not correlated. This suggests that the aucubin hydrolyzing β-glucosidase might not be under the control of the insect but potentially comes from gut microbiota or the host plant. The kinetic properties of the assayed β-glucosidase activity indicated simple Michaelis-Menten behavior for both substrates NPβGlc and aucubin. Our results clearly demonstrate that caterpillars actively decrease their total midgut β-glucosidase activity when feeding on plants that contain high amounts of detrimental glycosides. However, in no-choice situations, the down-regulation of β-glucosidase activity might not be sufficient to allow feeding on iridoid glycoside containing plants without suffering detrimental effects due to the (counterintuitive) increase in aucubin hydrolyzing β-glucosidase. Host plant switching as a behavioral adaptation might help generalist herbivores to circumvent the effects of either plant or gut microbiota that counteract adaption of insects to secondary plant compounds.

3.2 Introduction

Iridoid glycosides are secondary plant compounds that occur in over 50 plant families in many asterid clades (Albach et al., 2001). These compounds are cyclopentanoid monoterpene-derived compounds consisting of eight-, nine- or ten-carbon skeletons with an attached monosaccharide at C-1, normally β-D-glucose (Boros & Stermitz, 1990). Like other secondary plant compounds, iridoids have deterrent effects on non-adapted insects (Bernays & De Luca, 1981, Puttick & Bowers, 1988). In addition, iridoid glycosides often exert detrimental postingestive effects during larval development leading to a lower efficiency in converting ingested food, a lower relative growth rate, reduced larval weight, and a higher larval mortality (Bowers & Puttick, 1988, Puttick & Bowers, 1988, Stamp & Meyerhoefer, 2004). These toxic physiological effects are often dose-dependent and differ among individual iridoid glycosides depending on their chemical structure and the presence or absence of functional groups (Bernays & De Luca, 1981, Bowers & Puttick, 1988, Puttick & Bowers, 1988).

In general, to exert toxic effects, iridoid glycosides have to be activated by enzymes or non-enzymatically by acidic hydrolysis. To date, only the hydrolytic β-glucosidases (EC 3.2.1.21) have been shown to convert the non-reactive iridoid glycosides into highly reactive aglycones (Kim et al. 2000; Konno et al. 1999). The resulting iridoid aglycone is a dialdehyde that is similar to the structure of glutaraldehyde in aqueous solution (Bartholomaeus and Ahokas 1995). The mechanism of toxicity of iridoid glycosides is hypothesized to resemble the one of alkylating agents such as glutaraldehyde that bind covalently to nucleophilic side chains (e.g., $-NH_2$ of lysine) via imine formation (Bartholomaeus & Ahokas, 1995, Kim et al., 2000, Konno et al., 1997, Konno et al., 1999). Even though the protein interaction is non-selective (Wink, 2006), several studies show that after enzymatic hydrolysis, the iridoid aglycones cross-link proteins and also act as enzyme inhibitors depending on their chemical structure (Bartholomaeus & Ahokas, 1995, Konno et al., 1999, Ling et al., 2003, Park et al., 2007). In plants, β-glucosidases often co-occur with plant glycosides upon which they are specifically active (Morant et al., 2008, Zagrobelny et al., 2008, Zagrobelny et al., 2004). For some iridoid glycoside containing plants, the presence of β-glucosidases has been confirmed as well (Boonclarm et al., 2006, Konno et al., 1999, Mazzuca et al., 2006). β-Glucosidases are also present in insects, where they are mainly found as digestive enzymes in the gut (Terra & Ferreira, 1994, Terra & Ferreira, 2005). Several insect β-glucosidases that have been

biochemically characterized were shown to act upon a variety of substrates, including plant glycosides (Terra & Ferreira, 1994, Terra & Ferreira, 2005, Yu, 1989). Based on their relative catalytic efficiency towards several substrates, they are divided into the two classes A and B (Terra & Ferreira, 2005). Class B β-glucosidases include enzymes that are only active on substrates with hydrophobic aglycones, such as 4-nitrophenyl-glycosides and plant glycosides (Terra & Ferreira, 2005). Besides providing additional carbohydrates, their use of plant glycosides as substrates renders class B β-glucosidases important for plant-insect interactions (Terra & Ferreira, 2005). β-Glucosidases are supposed to interfere with the use of plants as potential hosts (Ferreira et al., 1997, Yu, 1989), as β-glucosidases may release toxic aglycones from digested plant glycosides that potentially lead to autoxicity in the insect. To render iridoid glycosides toxic, they need to be hydrolyzed by β-glucosidases. This makes insect β-glucosidases highly interesting candidates for studying adaptation to iridoid glycosides in insects as they might present the determining factor by which insects could control autotoxicity.

In this study, we tested the hypotheses that β-glucosidase activity plays a key role for insects in tolerating iridoid glycosides in their diet. One possible mechanism to control for the effects of toxic plant β-glycosides would be a specific regulation of the enzymes which are most active towards the detrimental plant glycosides. Some studies showed that, for several coleopteran and lepidopteran species, tolerance of toxic plant glycosides in their food was achieved by a specific reduction of β-glucosidase activity in the gut (Desroches et al., 1997, Ferreira et al., 1997, Lindroth, 1988, Pankoke et al., submitted). On the other hand, a reduced affinity of β-glucosidases for toxin releasing plant glycosides would present a biochemical adaptation, because this would also reduce the release of detrimental aglycones (Ferreira et al., 1997, Marana et al., 2000).

To investigate the underlying physiological mechanisms that might lead to tolerance of iridoid glycosides in the diet of generalist herbivorous insects, we used *Grammia incorrupta* (Lepidoptera, Arctiidae). Arctiids have been used as study species because they are, on the one hand, polyphagous; yet, on the other hand, they show specific adaptations to host plant chemistry (Conner, 2008). At least three classes of compounds, pyrrolizidine alkaloids, cardenolides, and iridoid glycosides, can be sequestered as anti-predator defenses (Bowers & Stamp, 1997, Hristov & Conner, 2005, Rothschild et al., 1979, Von Nickisch-Rosenegk et al., 1990, Von Nickisch-Rosenegk & Wink, 1993, Wink & Von Nickisch-Rosenegk, 1997).

As a larva, *G. incorrupta* is highly polyphagous, feeding on at least 80 species of (mostly herbaceous) flowering plant from at least 50 taxonomically widespread families (Singer, 2001). The larvae are true generalists, as individual caterpillars feed on 5 - 10 different plant species per day (Singer, 2001). However, they seem to prefer certain plant species over others, such as *Plantago* species (Plantaginaceae) (Singer & Stireman, 2001). Although the larvae switch very often between host plants in their natural habitat, larval survival and developmental rate on mixed-plant diets is rarely superior to performance on single-plant diets (Singer, 2001). When offered a choice of variously suitable host plants, larvae often select a diet that includes toxic plant species even if that impairs larval performance (Singer, 2001). By choosing acceptable but nutritionally inferior host-plant species that are rich in secondary plant compounds, *G. incorrupta* larvae increase their resistance to parasitoids (Singer et al., 2009, Singer & Stireman, 2003). When parasitized, *G. incorrupta* caterpillars exhibit pharmacophagy and the neurological response in gustatory-cells to secondary host plants changes (Bernays & Singer, 2005). This increases the preference of caterpillars for feeding on plants with toxic plant compounds and thereby, to sequester more toxic compounds like pyrrolizidine alkaloids and iridoid glycosides (Bernays & Singer, 2005). Food mixing in *G. incorrupta* is predominantly a negative behavioral response to plant secondary metabolites (Singer et al., 2002). This is consistent with the "toxin dilution" or "detoxification limitation hypothesis" (Freeland & Janzen, 1974), which states that dietary generalism helps animals to avoid autotoxicity. Whenever the pathway that detoxifies the formerly consumed toxic metabolite is saturated, insects respond by selecting different food plants that contain no or other toxins.

Being a food-mixing generalist, *G. incorrupta* larvae might possess a limited detoxification capacity towards iridoid glycosides. First, we wanted to test whether an iridoid glycoside containing plant would influence the larval development differently compared to a host plant without iridoid glycosides. Second, we wanted to know whether host plant chemistry would influence β-glucosidase activity in the larvae and thus, midgut β-glucosidase could mediate host plant use. For that, the larvae were reared on *Taraxacum officinale* (Asteraceae) and *Plantago lanceolata* (Plantaginaceae). These two plant species differ in their composition of secondary compounds. *T. officinale* contains sesquiterpene lactones and phenolic compounds (Schütz et al., 2006, Schütz et al., 2005, Williams et al., 1996) but does not contain iridoid glycosides. In comparison to *T. officinale*, *P. lanceolata* contains high amounts of iridoid glycosides, primarily aucubin and catalpol (Barton & Bowers, 2006, Rønsted et al., 2003;

Rønsted et al. 2000, Willinger & Dobler 2001). To address the biochemical basis of how the polyphagous *G. incorrupta* is able to feed on iridoid glycoside containing plants, we performed kinetic studies with the substrates NPβGlc and the iridoid glycoside aucubin to investigate the biochemical characteristics of *G. incorrupta* midgut β-glucosidases.

Overall, we designed this research to (1) find out how the caterpillars performed on an iridoid glycoside containing host plant in comparison to a plant without iridoids; (2) determine the substrate affinity of β-glucosidases towards NPβGlc and the iridoid glycoside aucubin; (3) determine midgut β-glucosidase activity of *G. incorrupta* larvae in relationship to the host plant chemistry of the plants; (4) test for a limited detoxification in *G. incorrupta* larvae when feeding on an iridoid glycoside containing plant; and (5) address the underlying mechanisms that could enables generalist insect herbivores to tolerate iridoid glycosides in their diet.

3.3 Materials and Methods

Insect feeding experiment

Eggs of *G. incorrupta* from a laboratory culture were kindly provided by Michael Singer (Wesleyan University, Middletown, Connecticut, USA). The larvae were reared at Hamburg University on an artificial diet (Bergomaz & Boppré, 1986) in a climate chamber at 26 °C until pupation. Eggs from several females from this culture were mixed randomly and resulting offspring was used for the feeding experiment. After hatching, all the larvae were fed with an artificial diet (Bergomaz & Boppré, 1986) for five days. Then, we divided the larvae into two groups of similar size. To allow habituation to the plant diet, we kept the caterpillars on the artificial diet from day five to eight and additionally offered to each group one of the host plants, *T. officinale* (Asteraceae) or *P. lanceolata* (Plantaginaceae). From day eight on, larvae were exclusively reared on the respective food plant that was used together with the artificial diet. Food was always provided *ad libitum*. Larvae were reared in plastic containers at 26 °C and a 16:8 h light:dark photoperiod. Host plants were collected at least twice a week from local populations and stored in a climate chamber at 16 °C until use. Larval weight was determined on day 21, 28, 42 and 49 (day 1 being the day when artificial diet and food plant were supplied together for the first time). To test the effects of host plant species on larval development time, once pupation began, we recorded the number of larvae that had pupated by seven days.

Enzyme preparation

To standardize for duration of larval development when we determined β-glucosidase activity, larvae reared on the two host plants were dissected on two consecutive days, on day 47 (*T. officinale*) and day 48 (*P. lanceolata*) after hatching. Before dissection, larvae were weighed to the nearest 0.1 mg. Then, larvae were dissected in cold 125 mM NaCl on ice (Ferreira et al., 1994). Midguts were isolated, freed from the peritrophic membrane and the food within and rinsed with 125 mM NaCl to remove the midgut contents completely. Using a glass Potter-Elvehjem tissue grinder, the gut sections were homogenized in double-distilled water while kept on ice. Then, midgut samples were frozen at -20 °C. To extract most of the β-glucosidase activity, which is mainly attached to the glycocalyx (Terra & Ferreira, 1994), the samples were frozen and thawed three times before centrifugation at 4 °C for 30 minutes at 25.000 g (Terra & Ferreira, 1994). The resulting supernatants were used as a source of β-glucosidase activity. For that, the supernatants were aliquoted and stored at -20 °C until use. The preparations could be stored for at least 6 months at -20 °C without any noticeable change in β-glucosidase activity (Pankoke, personal observation).

Protein determination and enzyme assays

Protein concentration of each sample was determined with Bradford reagent (Sigma Aldrich, Hamburg, Germany) by measuring the absorbance at 595 nm and comparing it to a standard curve of BSA (Bradford, 1976). To test for optimal reaction conditions, β-glucosidase activity was determined in citrate sodium phosphate buffer ranging from pH 6.0 to 7.0 in steps of 0.1. To measure class B β-glucosidase activity (Terra and Ferreira, 2005), we used 4-nitrophenyl-β-D-glucoside (NPβGlc) as a substrate which is hydrolyzed by class B β-glucosidases into glucose and the chromophore 4-nitrophenolate. All enzyme assays were performed at 30 °C in 100 mM citrate sodium phosphate buffer at pH 6.5 with NPβGlc (42 mM). Controls without enzyme extract were used to test for substrate autohydrolysis and controls without substrate were also included. The enzymatic reaction with NPβGlc was stopped by adding ice cold 0.5 M Na_2CO_3 solution. The absorbance of the released 4-nitrophenolate was measured at 400 nm (UV-Spectrophotometer Biochrom 2100 Pro) and the amounts of 4-nitrophenolate were calculated using appropriate standard curves (Lymar et al., 1995). The hydrolysis of aucubin (24 mM) by β-glucosidase was quantified by measuring the released glucose which is equimolar to the release of the aglycone aucubigenin. The reaction was stopped by heat denaturation of the enzyme in boiling water for 5 minutes. The amount of released glucose was determined with the coupling enzyme reaction using hexokinase and glucose-phosphate-

6-dehydrogenase according to Ogawa et al.(2001). Controls without substrate were included to measure the background of glucose, ATP and β-NAD within the enzyme extract. Controls without enzyme were included to test for aucubin autohydrolysis. To determine the reaction conditions for initial velocity, incubations were carried out for at least eight different periods of time (0 – 130 min) and twelve different protein concentrations. For all determinations of β-glucosidase activity in the experiments, the enzyme assays were performed under conditions such that the β-glucosidase activity was proportional to protein concentration and time. One unit of enzyme (U) is defined as the amount of enzyme that hydrolyzes one µmol of substrate per minute at 30 °C.

Kinetic properties of β-glucosidase activity

To determine the effect of different substrate concentrations on the β-glucosidase activity, midgut homogenates from larvae reared on *T. officinale* were tested (n = 3) using at least 16 substrate concentrations ranging from 0 mM to 100 mM for NPβGlc and at least nine different substrate concentrations of the iridoid glycoside aucubin (from 0 mM to 24 mM). Controls without substrate and controls without enzyme to test for substrate autohydrolysis were included for every substrate concentration.

Enzymatic activity that follows a steady state enzyme kinetic can be described by the Michaelis-Menten equation (1) (Copeland, 2000).

$$V = \frac{V_{\max} * C}{K_m + C} \qquad (1)$$

Here, V is the reaction velocity of the enzyme, V_{max} is the maximum reaction velocity of the enzyme, C is the substrate concentration used and K_m is the substrate concentration that provides a reaction velocity that is half of the maximal velocity obtained under saturating substrate conditions, named Michaelis constant (Copeland, 2000).

The Michaelis-Menten kinetic model was further modified to include two isoenzymes with differing Michaelis-Menten constants, in which both the kinetic of both β-glucosidases followed a simple Michaelis-Menten behavior as shown in equation (2).

$$V = \frac{V_{max1} * C}{K_{m1} + C} + \frac{V_{max2} * C}{K_{m2} + C} \qquad (2)$$

The experimental data were fitted to equation (1) and (2) and kinetic parameters were calculated with JMP 7.1 performing iterative non-linear regression analysis with a minimum of 5000 iterations.

Statistical analysis

Larvae died during the course of the experiment, therefore sample sizes varied substantially at different dates. Therefore, to evaluate the growth rates of larvae on the two host plants in our feeding experiment, we compared the weight of the larvae on four days during larval development with the Mann-Whitney U-test. To compare the influence of host plant species on the speed of larval development, we compared the number of pupae found within one week after the appearance of the first pupa using Fisher's Exact test.

Larval weight on the day of dissection of larvae reared on both host plants and their β-glucosidase activity determined with two substrates were tested for homogeneity of variance (Levene test) and normal distribution (Shapiro Wilk test). β-Glucosidase activity of larvae reared on both host plants and using the two different substrates were log10-transformed to obtain homogeneity of variance and normal distribution. To compare the effect of host plant species on the protein content of midgut homogenates and larval weight on the day of dissection, we used the t-test for equal variances. Additionally, we performed analysis of covariance (ANCOVA) to explain interindividual variation in NPβGlc hydrolyzing β-glucosidase activity (response variable), 'larval weight on the day of dissection' was used as the covariate and 'host plant' as the explanatory variable. To test for interaction between host plant and larval weight, we included the crossed term 'host plant x larval weight'. To test for correlations between the β-glucosidase activities towards the two substrates for larvae reared on either *T. officinale* or on *P. lanceolata*, we performed a simple linear correlation analysis and tested their significance by performing analysis of variance.

To compare the fit between the kinetic models, we performed an F-test for nested models as proposed by Motulsky and Christopoulos (2004). As the null hypothesis, the simpler model including fewer parameters is assumed to be correct. The F-test to compare the model fit between nested models is calculated as indicated below, using the sum of squares and the degrees of freedom from the computed curve statistics that result from the iterative nonlinear regression analysis.

$$F = \frac{(SS_1 - SS_2)/(DF_1 - DF_2)}{SS_2/DF_2}$$

An F-ratio near 1.0 means that the simpler model is correct, while a much greater F-ratio with a P-value lower than 0.05 implies that the more complicated model provides a significantly better fit than the simple model (Motulsky & Christopoulos, 2004). All statistical tests were performed with the statistical software JMP 7.1 (SAS) and Excel (Microsoft).

3.4 Results

Larval development and performance on two host plants

Larval weight was significantly less on *P. lanceolata* compared to those on *T. officinale* on all dates (Day 21, $U = 10.912$, Day 28, $U = 10.454$, Day 42, $U = -7.994$, Day 49, $U = 6.104$; $p < 0.0001$ in all analyses) (Figure 3.1).

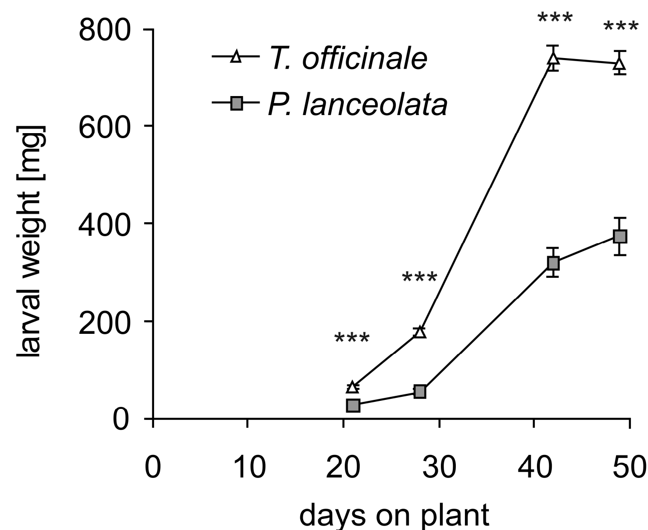

Figure 3.1 Larval weight of *Grammia incorrupta* larvae reared on *Taraxacum officinale* (white triangles) or on *Plantago lanceolata* (grey squares) was determined on four days during larval development and is shown as mean ± standard error. Significances are indicated by asterisks and correspond to $p < 0.0001$ for all comparisons. The number of larvae decreased over time – on day 21, $n_{T.\ officinale} = 129$ and $n_{P.\ lanceolata} = 139$; day 28, $n_{T.\ officinale} = 84$ and $n_{P.\ lanceolata} = 96$; on day 42, $n_{T.\ officinale} = 74$ and $n_{P.\ lanceolata} = 73$; and on day 48, $n_{T.\ officinale} = 43$ and $n_{P.\ lanceolata} = 50$. From day 42 on, larvae feeding on *T. officinale* began to pupate.

We also found significant differences in the duration of larval development of the *G. incorrupta* caterpillars reared on the two plants (Fisher Exact test, two-tailed, $p < 0.0003$; Figure 3.2). Significantly more larvae reared on *T. officinale* than on *P. lanceolata* started to pupate between day 43 and 49 (Fisher Exact test, left-sided, $p < 0.0002$; Figure 3.2). Larval mortality did not differ between the two plant species when we compared the number of larvae still alive on the four days when larval weight was determined.

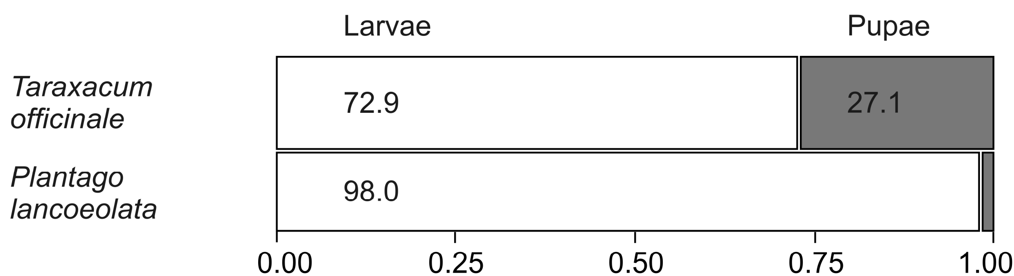

Figure 3.2 The mosaic plot for *Grammia incorrupta* larvae (white bar) and pupae (grey bar) that were either reared on *Taraxacum officinale* (n = 59) or on *Plantago lanceolata* (n = 51). Percent values are given for the number of pupae formed within the week following the formation of the first pupa and the numbers of the remaining larvae on either of the two host plants.

Kinetic properties of β-glucosidase activity

Kinetic analysis of the β-glucosidase of *G. incorrupta* larvae that were reared on *T. officinale* using the synthetic substrate NPβGlc revealed Michaelis-Menten behavior for substrate concentrations between 0 mM and 100 mM. The β-glucosidase activity of all three individuals is shown in the Eadie-Hofstee plot (Figure 3.3).

We performed iterative nonlinear regression analysis to obtain best fit values for V_{max} and K_m for equation (1) for one enzyme and (2) for two enzymes. Next, we compared the curve fit of equation (1) with the curve fit of equation (2) using an F-test for nested models (see Materials and Methods). The F-test showed that the *G. incorrupta* larvae reared on *T. officinale* must have two major midgut β-glucosidases with different Michaelis constants for NPβGlc (Table 3.1). One β-glucosidase had a higher substrate affinity towards NPβGlc while the other β-glucosidase had a lower substrate affinity towards NPβGlc. The β-glucosidase ratio of the maximal velocity between the two enzymes (higher substrate affinity versus lower affinity) was rather stable for all three individuals and was in the range of 1.3 to 2.0. For *G. incorrupta* larvae reared on *P. lanceolata*, kinetic analysis of the β-glucosidase activity

towards the iridoid glycoside aucubin as substrate revealed Michaelis-Menten behavior for substrate concentrations between 0 mM and 24 mM. The results of the F-statistic indicate that *G. incorrupta* larvae have only one β-glucosidase that is active against aucubin (Table 3.1).

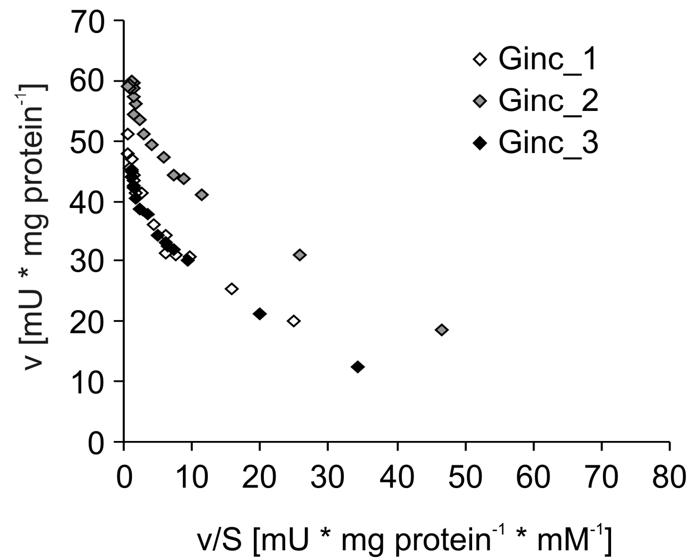

Figure 3.3 Eadie-Hofstee plot of *Grammia incorrupta* β-glucosidase activity of three larvae (white, grey and black diamonds) reared on *Taraxacum officinale*. The specific β-glucosidase activity [v] determined with different concentrations of 4-nitrophenyl-β-D-glucoside was plotted against the β-glucosidase activity per substrate concentration [v/S]. The points of every curve are the means of two independent determinations.

Table 3.1 Kinetic parameters (estimated means + S.E.) of *Grammia incorrupta* β-glucosidase activity of individual larvae either reared on *Plantago major* or *P. lanceolata* for the substrates 4-nitrophenyl-β-D-glucoside (NPβGlc) and aucubin. Kinetic parameters were determined by iterative nonlinear regression based on the Michaelis-Menten-model (1) or the Michaelis-Menten-model including two isoforms with different K_m and V_{max} (2). P-values < 0.05 indicate that the more complex model (2) fits significantly better than the simpler model (1).

No	Host plant	Substrate	K_{m1} [mM]	V_{max1} [mU mg protein^{-1}]	K_{m2} [mM]	V_{max2} [mU mg protein^{-1}]	model	F-test	P-value
1	*P. major*	NPβGlc	0.43 ± 0.15	29.04 ± 3.84	12.95 ± 5.97	21.74 ± 2.84	(1) vs. (2)	$F_{2,14} = 36.9$	$P < 0.001$
2	*P. major*	NPβGlc	0.53 ± 0.08	42.24 ± 2.88	15.77 ± 5.76	21.32 ± 2.19	(1) vs. (2)	$F_{2,16} = 64.1$	$P < 0.001$
3	*P. major*	NPβGlc	0.46 ± 0.09	29.17 ± 2.73	12.40 ± 4.55	18.51 ± 2.17	(1) vs. (2)	$F_{2,18} = 55.7$	$P < 0.001$

No	Host plant	Substrate	K_{m1} [mM]	V_{max1} [mU mg protein^{-1}]	K_{m2} [mM]	V_{max2} [mU mg protein^{-1}]	model	F-test	P-value
1	*P. lanceolata*	Aucubin	3.92 ± 0.90	2.27 ± 0.18	---	---	(1) vs. (2)	$F_{1,8} = 0.0$	n.s.
2	*P. lanceolata*	Aucubin	1.35 ± 0.35	2.87 ± 0.16	---	---	(1) vs. (2)	$F_{1,8} = 0.00$	n.s.
3	*P. lanceolata*	Aucubin	1.88 ± 0.33	9.56 ± 0.36	---	---	(1) vs. (2)	$F_{2,18} = 3.56$	n.s.

For the determination of β-glucosidase activity, three different individuals reared on *Taraxacum officinale* were examined using at least 16 different substrate concentrations of NPβGlc (0-100 mM), while three other individuals reared on *Plantago lanceolata* were tested using at least nine different substrate concentrations of the iridoid glycoside aucubin (0-24 mM); the enzyme assays were all performed in 100 mM sodium citrate-phosphate buffer at pH 6.5, and 30 °C.

Enzyme assays

β-Glucosidase activity of *G. incorrupta*, when measured under standard conditions in 100 mM citrate sodium phosphate buffer at 30 °C showed linearity with respect to time (up to 60 minutes) and with respect to protein concentrations (up to 70µg/mL). The β-glucosidase of *G. incorrupta* measured with NPβGlc has a maximum activity at pH 6.5. Between pH 6.0 and pH 7.0, the β-glucosidase retains between 85% and 100% of the maximum activity.

When we compared the protein content of the midgut homogenates of *G. incorrupta* larvae reared on the two host plants, we found that the protein concentrations of midgut homogenates did not differ between larvae reared on the two host plants (T-test, $t_{1,19}$= 1.219, p = 0.239). In addition, we tested whether the larval weight on the day of dissection depended on the host plant, and we only found a tendency for larvae being slightly lighter when feeding on plantain (T-test, $t_{1,19}$= 1.774, p = 0.093).

Next, we compared the specific midgut β-glucosidase activity per mg protein between the two host plants using an ANCOVA. We included 'larval weight on the day of dissection' as the covariate and to test for interaction between the covariate and the main effect, we included the crossed term 'host plant x larval weight'. We found highly significant differences in β-glucosidase activity between larvae reared on the two host plants and the activity was differentially influenced by larval weight depending on the host (ANCOVA, $F_{3,19}$ = 20.160, $p < 0.0001$, Figure 3.4). When the larvae were reared on *P. lanceolata*, the β-glucosidase activity towards NPβGlc was significantly reduced in comparison to the activity of the larvae reared on *T. officinale* (F-test, $F_{1,1}$ = 38.377, $p < 0.0001$; Figure 3.4). The covariate 'larval weight' alone was not significantly correlated with the β-glucosidase activity (F-test, $F_{1,1}$ = 1.288, p = 0.273). However, when we compared the larval weight in relationship to the host plant, the correlation between β-glucosidase activity and larval weight was positive when the larvae were reared on *T. officinale*, yet it was negative when the larvae were reared on *P. lanceolata* (F-test, $F_{1,1}$ = 8.039, $p < 0.012$; Figure 3.5a and Table 3.2).

Using the iridoid glycoside aucubin as substrate, we found highly significant differences in β-glucosidase activity between larvae reared on the two host plants and the β-glucosidase activity was correlated with larval weight on both host plants (ANCOVA, $F_{3,19}$ = 7.862, p = 0.002). We detected a very low β-glucosidase activity towards aucubin in the larvae reared on *T. officinale* and found a significantly higher β-glucosidase activity towards aucubin

when the larvae were reared on *P. lanceolata* (F-test, $F_{1,1} = 8.664$, $p < 0.010$; Figure 3.4). The β-glucosidase activity towards aucubin was significantly negatively correlated with larval weight (F-test, $F_{1,1} = 5.796$, $p = 0.029$), yet in this case larvae on both plants showed the same pattern (F-test, $F_{1,1} = 0.223$, $p = 0.643$; Figure 3.5b and Table 3.2).

When we tested whether β-glucosidase activity against NPβGlc and aucubin were correlated, we found no correlation for either host plant (*T. officinale*: $r^2 = 0.145$, $F_{1,8} = 1.361$, $p = 0.277$; *P. lanceolata*: $r^2 = 0.168$, $F_{1,8} = 1.616$, $p = 0.239$).

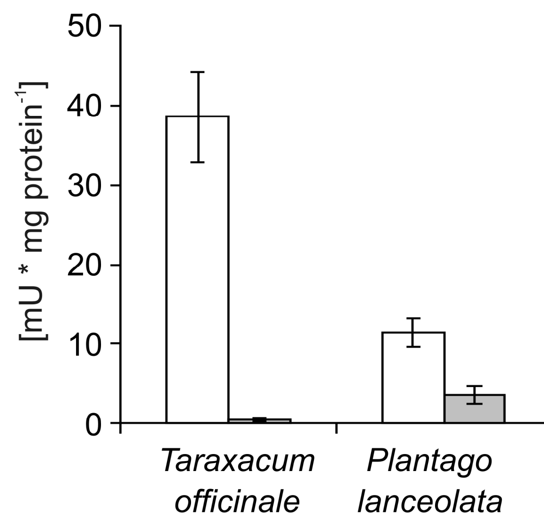

Figure 3.4 The β-glucosidase activity (means + S.E.) of *Grammia incorrupta* last instar larvae reared on *Taraxacum officinale* (without iridoid glycosides) (n = 10) or on *Plantago lanceolata* (high iridoid glycoside content) (n = 10). The β-glucosidase activity was determined with 42 mM 4-nitrophenyl-β-D-glucoside (white bars) and 22 mM aucubin (grey bars).

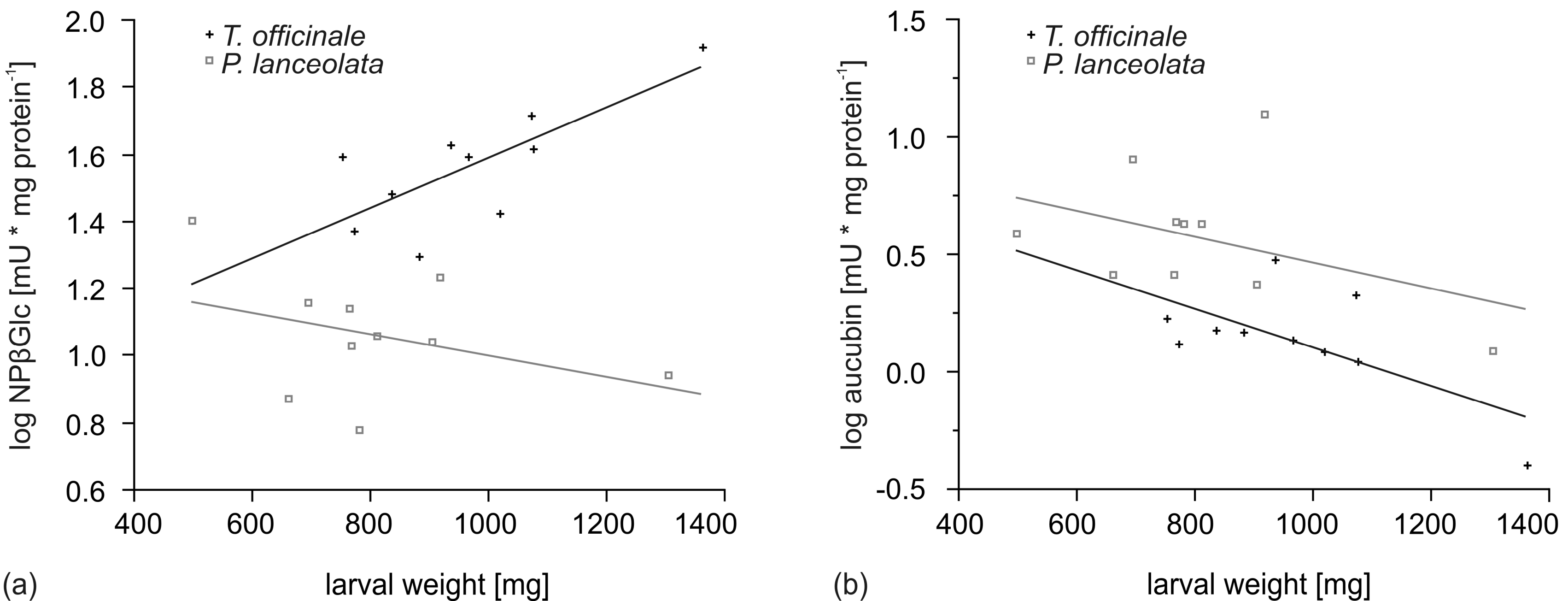

Figure 3.5 (a) and (b) Log-transformed β-glucosidase activity of *Grammia incorrupta* last instar larvae reared on *Taraxacum officinale* (without iridoid glycosides) (n = 10) or *Plantago lanceolata* (high iridoid glycoside content) (n = 10). The β-glucosidase activity was plotted against the larval weight on the day of dissection. (a) The correlation of β-glucosidase activity determined with 42 mM 4-nitrophenyl-β-D-glucoside and larval weight differs significantly between the host plants and is positive for larvae reared on *T. officinale* (black crosses) and negative for larvae reared on *P. lanceolata* (grey squares). (b) The β-glucosidase activity determined with 22 mM aucubin is negatively correlated with larval weight of larvae reared on both hosts.

Table 3.2 Analysis of covariance to explain interindividual variation in β-glucosidase activity determined with 4-nitrophenyl-β-D-glucoside (NPβGlc) and aucubin in relationship to larval weight on the day of dissection of *Grammia incorrupta* larvae that were reared on two host plants, either *Taraxacum officinale* or *Plantago lanceolata*.

Source of variation	**df**	**SS**	**F**	**P (power)**
NPβGlc				
ANCOVA – total model	3	1.456	20.160	< 0.0001
ANCOVA – Error	16	0.385		
Host plant	1	0.924	38.377	< 0.0001
Larval weight on dissection day	1	0.031	1.288	0.273
Host plant x Larval weight (dissection day)	1	0.194	8.039	0.012
Aucubin				
ANCOVA – total model	3	1.276	7.862	< 0.0001
ANCOVA – Error	16	0.866		
Host plant	1	0.469	8.664	0.010
Larval weight on dissection day	1	0.314	5.796	0.029
Host plant x Larval weight (dissection day)	1	0.012	0.223	0.643

3.5 Discussion

The larval development of the arctiid herbivore *G. incorrupta* was significantly faster when the caterpillars were reared on *T. officinale* (no iridoids) in comparison to the iridoid glycoside containing *P. lanceolata*. The iridoid glycosides present in *P. lanceolata* have previously been shown to significantly decrease larval growth rates in generalist herbivores (Bowers & Puttick, 1988, Gange & West, 1994, Puttick & Bowers, 1988). A slow growth rate, which in consequences leads to a longer larval development and a later pupation, may present high fitness costs for caterpillars in the field. The longer the larval development, the higher the probability of *G. incorrupta* larvae dying from parasitism, which typically increases over the course of the season (Singer, 2001). On the other hand, *G. incorrupta* larvae are known to sequester iridoid glycosides to protect themselves against parasites (Bowers, 2008, Singer & Bernays, 2008) which might compensate for the prolonged larval development when feeding on *P. lanceolata*. It seems that different *Plantago* species have a different impact on larval development, as larval performance on *P. insularis* (Singer, 2001) was more similar to larval performance on *T. officinale* than *P. lanceolata* in our no-choice experiments. This could be due to qualitative or quantitative differences in iridoid glycosides levels in *P. insularis*, as iridoid glycosides are known to differ in their effect depending on their chemical structure and quantity (Bernays & De Luca, 1981, Bowers & Puttick, 1988, Nishida & Fukami, 1989, Puttick & Bowers, 1988).

To account for differences within our sampled population, we determined Michaelis constants of *G. incorrupta* midgut β-glucosidase activity for three individual larvae that were reared on *T. officinale* or on *P. lanceolata*. Using the artificial substrate NPβGlc, we detected two β-glucosidases in larval midguts of larvae reared on *T. officinale* whose kinetics showed simple Michaelis-Menten behavior. Consistent for all three individuals, we found a β-glucosidase with a high affinity towards the substrate NPβGlc, comparable to other biochemically characterized and purified lepidopteran midgut β-glucosidases (Azevedo et al., 2003, Marana et al., 2000, Pratviel-Sosa et al., 1987, Santos and Terra 1985). Typical Michaelis constants of insect β-glucosidases range between 0.2 - 2.0 mM for NPβGlc (Terra & Ferreira, 1994). In addition, we found a second β-glucosidase with a much lower affinity towards NPβGlc. The existence of a β-glucosidase with such a low Michaelis constant has been reported only once before for the coleopteran species *Sitophilus oryzae* (Baker & Woo, 1992). We did not purify the enzymes from the midgut homogenates which might

explain the variability and the standard errors of the low affinity Michaelis constants. Therefore, the lower affinity constant should be taken as an approximation to the true K_m value. Using aucubin as substrate, we found one β-glucosidase in larvae reared on *P. lanceolata*. The Michaelis constants for aucubin varied more compared to NPβGlc, but this could be due to the relatively lower β-glucosidase activity that made it more difficult to determine the Michaelis constant.

Next, we wanted to test whether β-glucosidase activity in *G. incorrupta* larvae was differently influenced by host plant chemistry. Determining the total class B β-glucosidase activity using NPβGlc, we found a significantly lower activity per mg protein when the larvae were reared on the iridoid glycoside containing host plant *P. lanceolata*. Furthermore, we could show that the lower the β-glucosidase activity in the larvae reared on plantain was the more the larvae weighed on the day of dissection (which was day 48 after hatching). This strongly suggests that the decrease of β-glucosidase activity in the larvae presents a mechanism for tolerating toxic plant glycosides in the diet. In consequence, larvae with a better regulation of β-glucosidase activity might gain more weight and develop faster on *P. lanceolata*. However, larval performance on the iridoid glycoside containing *P. lanceolata* was significantly impaired for most of the larvae in comparison to the larval performance on the iridoid glycoside-free *T. officinale* (Figure 3.1). The differences in larval performance indicate that *G. incorrupta* caterpillars have a limited capacity to feed solely on iridoid glycoside containing host plants without suffering from detrimental effects.

To test the hypothesis that *G. incorrupta* caterpillars have a limited detoxification capacity or rather an increased risk of autotoxicity, we used the iridoid glycoside aucubin as a β-glucosidase substrate. By hydrolyzing aucubin, β-glucosidase liberates the toxic aglycone aucubigenin which is known to strongly reduce the nutritive value of plant foliage by unselectively forming high-molecular protein complexes and by decreasing the lysine content in proteins (Konno et al., 1999). And indeed, we found a significantly induced β-glucosidase activity towards aucubin in the larvae reared on *P. lanceolata*. The significant negative correlation between the β-glucosidase activity towards aucubin and larval weight on the day of dissection for larvae reared on either *P. lanceolata* or *T. officinale* strongly suggests that the aucubin hydrolyzing β-glucosidase measured in *G. incorrupta* larvae might be the cause for the significantly reduced larval growth rate on plantain. Thus, we can conclude that larvae have a limited detoxification capacity towards iridoid glycosides. However, the induction of

the aucubin hydrolyzing β-glucosidase in the larvae feeding on plantain seemed to be rather counterintuitive compared to the observed decrease of the NPβGlc hydrolyzing β-glucosidase. As β-glucosidase activity towards NPβGlc and aucubin are not correlated, this indicates that the β-glucosidase that hydrolyzes aucubin is probably not the same as the NPβGlc hydrolyzing β-glucosidase. Thus, the induction of the aucubin-hydrolyzing β-glucosidase might not be an inadequate reaction of *G. incorrupta* larvae to *P. lanceolata*, but might occur because the aucubin-hydrolyzing β-glucosidase was produced by the host plant itself or the gut microbiota of *G. incorrupta*. To exclude β-glucosidases of plant origin in our enzyme assay, we removed the peritrophic membrane during dissection and rinsed the midgut tissue of the larvae thoroughly before homogenizing it. Therefore, it seems rather improbable that aucubin-hydrolyzing β-glucosidases are of plant origin and not coming from the tissue itself or from its surface.

Apart from the enzymatic equipment of the herbivore itself, other trophic levels might influence the biochemical and physiological adaptation of a generalist herbivore to different host plants. To circumvent the negative effects occurring by the hydrolysis of iridoid glycosides, host plant switching might present a potential behavioral adaptation in the larvae of *G. incorrupta*. Generalists are thought to possess a limited detoxification capacity in comparison to specialized herbivores, so that host plant switching helps to dilute detrimental concentrations of toxic plant compounds in the diet that otherwise might lead to self-intoxication (Freeland & Janzen 1974, Marsh et al. 2006, Singer et al. 2002). Our results demonstrate that without host plant switching, *G. incorrupta* larvae feeding on *P. lanceolata* react by reducing their β-glucosidase activity. Regardless of the regulation of the NPβGlc hydrolyzing β-glucosidase activity, most larvae feeding on *P. lanceolata* showed reduced performance. As the negative correlation of larval weight and β-glucosidase activity towards aucubin indicate for either host plant, the reduced performance of larvae reared on plantain is most probably caused by the aucubin hydrolyzing β-glucosidase which might not be under the control of the caterpillars but could stem from the plant or gut microbes. In the context of tolerance towards iridoid glycoside containing host plants, our results strongly support the hypothesis that host plant switching in *G. incorrupta* larvae might be a behavioral adaptation to decrease autotoxicity based on the potentially uncontrollable aucubin-hydrolyzing β-glucosidase. Because of the specific toxicity of iridoid glycosides, host plant switching might serve to balance nutrient uptake in addition to counteracting the reduced intake of digestible protein and essential amino acids. Based on the biochemical and physiological

analysis of β-glucosidase activity, our data suggest that the behavioral adaptation of host plant switching in generalist insects might be best explained by the detoxification limitation hypothesis and allows for tolerance of iridoid glycosides in generalist insect herbivores.

Concerning the aucubin-hydrolyzing β-glucosidase, we can not rule out that it was produced by gut microbes of the larvae. Symbiotic yeasts, fungi and bacteria have been reported for many different insect orders (Dowd, 1992, Janson et al., 2008, Saffo, 1992). It has also been suggested that the intestinal microbiota produces either detoxifying enzymes or otherwise toxic metabolites from secondary plant compounds (Dillon & Dillon, 2004, Dowd, 1992, Genta et al., 2006, Spiteller et al., 2000). Detoxification enzymes as well as toxic metabolites are thought to further influence the metabolism of the insect host and its ability to use host plants that differ in plant allelochemicals (Dowd, 1992, Ince et al., 2008, Janson et al., 2008). In the few lepidopteran species that have been studied, some of the gut endosymbionts have been identified (Brinkmann et al., 2008, Broderick et al., 2004, Inglis et al., 2000) and further characterized (Ince et al., 2008). Some of the bacteria found in the pine processionary moth, *Thaumetopoea pityocampa,* were shown to metabolize typical β-glucosidase substrates like plant glycosides (Ince et al., 2008). Some *Bacillus* species (Okstad et al., 1999, Papalazaridou et al., 2003) and among them the important insect pathogen, *Bacillus thuringiensis,* are known to possess a β-glucosidase that is active against the iridoid glycoside aucubin (Baden & Dobler, 2009). Several *Bacillus* species were found in lepidopteran guts potentially ingested by feeding herbivores. For the gypsy moth *Lymantria dispar*, Broderick et al. (2004) could show that feeding on different host plants substantially altered the microbial composition of the midguts. In our no-choice feeding experiment, we impaired the natural behavior of host plant switching in *G. incorrupta* larvae by providing only one host plant (Singer, 2001, Singer & Stireman, 2001). This may have affected the presence and composition of the microbial community in *G. incorrupta* guts that were able to use the iridoid glycosides as carbon source by producing iridoid glycoside hydrolyzing β-glucosidase. Nevertheless, to attribute the aucubin-hydrolyzing β-glucosidase to the larvae, the plant or gut microbes, further studies have to be undertaken.

4 How are insect herbivores able to feed on iridoid glycoside containing host plants? Comparison of the generalist *Estigmene acraea* (Arctiidae) and the specialist *Junonia coenia* (Nymphalidae)

4.1 Summary

Iridoid glycosides are secondary plant compounds with deterrent and post-digestive detrimental effects on non-adapted herbivores. Iridoid glycosides are biochemically activated by β-glucosidase which then leads to the observed detrimental effects. Nevertheless, certain generalist and specialist insects use iridoid glycoside containing plants as hosts. In this study, we compared the β-glucosidase activity of two lepidopteran species: the oligophagous iridoid glycoside specialist, *Junonia coenia* (Nymphalidae), and the polyphagous generalist, *Estigmene acraea* (Arctiidae). We reared the larvae on two iridoid glycoside containing host plants that differ in their iridoid glycoside concentration, *Plantago major* (low iridoids) and *P. lanceolata* (high iridoids). In both lepidopteran species, we determined β-glucosidase activity with the standard substrate 4-nitrophenyl-β-D-glucoside and the iridoid glycoside aucubin. Whereas the generalist *E. acraea* and the specialist *J. coenia* did not differ in their β-glucosidase activity (determined with 4-nitrophenyl-β-D-glucoside) when the larvae were reared on *P. major*, the generalist *E. acraea* had a significantly lower β-glucosidase activity than the specialist *J. coenia* towards 4-nitrophenyl-β-D-glucoside when the larvae were reared on *P. lanceolata*. Additionally, we found an aucubin hydrolyzing β-glucosidase in both species. The β-glucosidase activity towards aucubin was slightly higher in the specialist on *P. major*, but did not differ between the generalist and the specialist when the larvae were reared on *P. lanceolata*. In both species, the β-glucosidases active towards aucubin had similar Michaelis constants ranging from 1.2 mM to 1.8 mM. Based on these results, we conclude that neither for the specialist nor for the generalist, can β-glucosidase be the key enzyme that allows adaptation to iridoid glycoside containing plants. We discuss possible other means that might allow these insects to cope with iridoid glycosides in their host plants.

4.2 Introduction

Iridoid glycoside containing plants have been studied over the last twenty years with regard to chemical defense and feeding specialization in insects. Iridoids have deterrent effects on non-adapted herbivores, while they serve as feeding stimulants, oviposition cues and chemical defenses specialist herbivores (Bowers, 1983, Bowers, 1984, Bowers, 1991, Pereyra & Bowers, 1988). However, very little is known about the physiological and biochemical adaption of insects to iridoid glycoside containing host plants. Hundreds of iridoid glycosides, inherent to several asterid clades (Albach et al., 2001, Waterman, 2007), have been characterized, and they all share a basic chemical structure. Iridoids consist of a monoterpene moiety that is usually linked via a β-glycosidic bond to a carbohydrate which is glucose in most cases (Sampaio-Santos & Kaplan, 2001). Various studies have shown that iridoid glycosides are biochemically rather inactive as long as they have not been hydrolyzed by β-glucosidase (EC 3.2.1.21) (Bartholomaeus & Ahokas, 1995, Kim et al., 2000, Konno et al., 1999). β-Glucosidases are ubiquitous enzymes occurring in Eukarya, Archaea, and Bacteria (Henrissat et al., 1995, Stick & Williams, 2009). In plants that rely on glycosides as chemical defense, β-glucosidases were shown to co-occur alongside the specific plant glycosides upon which they are active (Konno et al., 1999, Morant et al., 2008). Although in some insect species, β-glucosidases were found in defensive glands and even in the hemolymph (Franzl et al., 1989, Soetens et al., 1993), usually, insect β-glucosidases are found in the digestive tract where they function as digestive enzymes (Terra & Ferreira, 1994, Terra & Ferreira, 2005, Terra et al., 1996). Due to their wide substrate specificity for β-D-glucosides, β-glucosidases are thought to play a crucial role in herbivorous insects when adapting to plant glycosides in their diet (Terra & Ferreira, 2005).

To test this hypothesis, lepidopterans are excellent study organisms. Within this insect order, all degrees of food specialization from broad generalists to strictly monophagous specialists that are highly adapted to specific secondary plant compounds can be found. Food specialization in lepidopterans due to key innovations is thought to have driven the diversification of lepidopterans with respect to many secondary plant compounds in the dicotyledons (Ehrlich & Raven, 1964, Wheat et al., 2007). The key innovations thus far reported in some lepidopteran taxa are clade-specific enzymes. These enzymes are able to

detoxify specific secondary plant compounds and, in turn, may have led to coevolution between the lepidopterans and their host plants (Berenbaum, 1983, Li et al., 2003, Wheat et al., 2007).

To compare how the biochemical adaptation to iridoid glycosides might differ between an iridoid glycoside specialist and a generalist species, we chose the well studied iridoid glycoside specialist *Junonia coenia* (Nymphalidae) and the arctiid saltmarsh caterpillar *Estigmene acraea* (Arctiidae). *Junonia coenia* caterpillars feed solely on iridoid glycoside containing plants and are known to store iridoid glycosides in their hemolymph in concentrations up to 25% of their total dry weight (Bowers, unpublished data, Bowers & Collinge, 1992, Strohmeyer et al., 1998). The sequestration of iridoid glycosides serves the caterpillars as an anti-predator defense (Bowers & Collinge, 1992, Theodoratus & Bowers, 1999). In contrast, the caterpillars of *E. acraea* are highly polyphagous generalists that feed on different host plants on a daily basis (Bernays et al., 2004, Cates, 1981, Singer & Bernays, 2008). For *E. acraea*, food mixing was shown to provide increased larval fitness against parasitoids due to the sequestration of toxins from low-quality food plants (Singer et al., 2004). In our feeding experiment, we reared both species on one of two *Plantago* species that differed in their iridoid glycoside concentration: *P. major* contains low concentrations of aucubin as the major iridoid glycoside (Barton & Bowers, 2006, Rønsted et al., 2003, Willinger & Dobler, 2001), while *P. lanceolata* contains high concentrations of mainly aucubin and catalpol (Barton & Bowers, 2006, Rønsted et al., 2003, Rønsted et al., 2000, Willinger & Dobler, 2001).

We designed this study to address the question of whether β-glucosidase might be the key enzyme important in tolerating dietary iridoid glycosides in these two species. To compare the metabolism of the generalist with that of the iridoid specialist, we (1) determined the substrate affinity of caterpillar β-glucosidases towards the substrate 4-nitrophenyl-β-D-glucoside and the iridoid glycoside aucubin; (2) performed heat inactivation experiments to test for one versus two isoforms of β-glucosidase; (3) determined total midgut β-glucosidase activity of the larvae of both species in relationship to the host plant chemistry of the plants by using the two substrates mentioned above; (4) suggest mechanisms by which these generalist and specialist herbivores tolerate iridoid glycosides in their diet.

4.3 Materials and Methods

Insect rearing and feeding experiment

Eggs of *Junonia coenia* and *Estigmene acraea* were derived from a lab culture maintained in the Bowers lab at the University of Colorado. The larvae were reared in plastic containers in a climate chamber at 22 °C and a 16:8 h light:dark photoperiod. They were reared on either *Plantago lanceolata* (Plantaginaceae) or on *P. major* until they reached the last larval instar. Food was always provided *ad libitum*. Host plants were collected once to twice a week from local populations in Hamburg and stored in the refrigerator at 4 °C until use.

Enzyme preparation

Before dissection, larvae were weighed to the nearest 0.1 mg. Then, larvae were dissected in cold 125 mM NaCl on ice (Ferreira et al., 1994). Midguts were isolated and rinsed to remove the gut contents. The gut sections were then homogenized in cold 50% 10 mM NaCl PBS/ 50% glycerol, pH 7.4 with a glass Potter-Elvehjem tissue grinder. Then, all samples were centrifuged at 4 °C for 45 minutes at 10,000 g and the resulting supernatants were collected and used as a source of enzymes. The enzyme preparations could be stored for at least three years at -80 °C without any noticeable change in β-glucosidase activity (Pankoke, personal observation).

Protein determination and enzyme assays

Protein concentration of each sample was determined with Bradford reagent (Sigma Aldrich, Hamburg, Germany) by measuring the absorbance at 595 nm and comparing it to a standard curve of BSA (Bradford, 1976). To test for optimal reaction conditions, β-glucosidase activity was determined in citrate sodium phosphate buffer with a pH ranging from 6.0 to 7.0, in steps of 0.1. To measure class B β-glucosidase activity (Terra and Ferreira, 2005), we used 4-nitrophenyl-β-D-glucoside (NPβGlc) as a substrate, which is hydrolyzed by class B β-glucosidases into glucose and the chromogenic 4-nitrophenolate. All enzyme assays were performed at 30 °C in 100 mM citrate sodium phosphate buffer at the optimal pH for the two species. This was pH 6.7 for *E. acraea*, whereas the assays for *J. coenia* were performed at pH 6.6.

For the substrate NPβGlc, the substrate concentration was 5 mM. Controls without enzyme extract to test for substrate autohydrolysis, as well as controls without substrate, were included. The enzymatic cleavage of NPβGlc was stopped by adding ice cold 0.5 M Na_2CO_3 solution. The absorbance of the released 4-nitrophenolate was measured at 400 nm (UV-Spectrophotometer Biochrom 2100 Pro) and the corresponding amount of 4-nitrophenolate calculated using appropriate standard curves (Lymar et al., 1995). The hydrolysis of aucubin (5 mM) by β-glucosidase was quantified by measuring the released glucose which is equimolar to the release of the aglycone aucubigenin. After the desired period of incubation (ranging from 60 to 120 min, see below), the reaction was stopped by denaturing the enzyme in boiling water for 5 minutes. The amount of released glucose was determined with the coupling enzyme reaction using hexokinase and glucose-phosphate-6-dehydrogenase according to (Ogawa et al., 2001). Controls without substrate were included to measure the background of glucose, ATP and β-NAD in the enzyme extract. Controls without enzyme were included to test for aucubin autohydrolysis. To determine the reaction conditions for initial velocity, incubations were carried out for at least eight different periods of time (0 - 120 min) and eight different protein concentrations. For all determinations of β-glucosidase activity in the experiments, the enzyme assays were performed under conditions such that the β-glucosidase activity was proportional to protein concentration and time. One unit of enzyme (U) is defined as the amount of enzyme that hydrolyzes one µmol of substrate per minute at 30 °C.

Kinetic properties of β-glucosidase activity

To determine the effect of different substrate concentrations on the β-glucosidase activity, midgut homogenates of *J. coenia* larvae were tested using at least eight different substrate concentrations ranging from 0.4 mM to 5.6 mM (n = 1, larvae reared on *P. major* and n = 1, larvae reared on *P. lanceolata*) or from 0 mM to 32 mM (n = 3, larvae reared on *P. lanceolata*) for NPβGlc and nine different substrate concentrations of the iridoid glycoside aucubin from 0 mM to 18 mM (n = 3, larvae reared on *P. lanceolata*). For *E. acraea*, we used at least 12 substrate concentrations in the range from 0 mM to 80 mM for NPβGlc (n = 3, larvae reared on *P. major*) and nine different substrate concentrations of the iridoid glycoside aucubin from 0 mM to 18 mM (n = 3, larvae reared on *P. lanceolata*). Controls without substrate and controls without enzyme extract to test for substrate autohydrolysis were included for every substrate concentration.

Enzymatic activity that follows a steady state enzyme kinetic can be described by the Michaelis-Menten equation (1) (Copeland, 2000).

$$V = \frac{V_{\max} * C}{K_m + C} \qquad (1)$$

Here, V is the reaction velocity of the enzyme, V_{max} is the maximum reaction velocity of the enzyme, C is the substrate concentration used and K_m is the substrate concentration that provides a reaction velocity that is half of the maximal velocity obtained under saturating substrate conditions, named Michaelis constant (Copeland, 2000).

The Michaelis-Menten kinetic model was further modified to include two isoenzymes with differing Michaelis-Menten constants, in which the kinetics of both β-glucosidases follow simple Michaelis-Menten behavior as shown in equation (2).

$$V = \frac{V_{max1} * C}{K_{m1} + C} + \frac{V_{max2} * C}{K_{m2} + C} \qquad (2)$$

At higher substrate concentrations, substrate inhibition can cause nonhyperbolic behavior of the enzymatic activity. Here, a second molecule of substrate can bind to the enzyme-substrate complex to form an inactive ternary, substrate-enzyme-substrate complex. Substrate inhibition can be described by equation (3),

$$V = \frac{V_{\max} * C}{K_m + C + \frac{C^2}{Ki}} \qquad (3)$$

where the term K_i represents the dissociation constant for the inhibitory substrate-enzyme-substrate ternary complex (Copeland, 2000).

The experimental data were fitted to equation (1), (2), or (3) respectively and kinetic parameters were calculated with JMP 7.1 performing iterative non-linear regression analysis with a minimum of 800 iterations.

Heat inactivation experiments

To calculate thermal inactivation constants of β-glucosidase activity, midgut extracts of *J. coenia* larvae reared on *P. lanceolata* ($n = 3$) were incubated in 0.1 M citrate sodium phosphate buffer at pH 6.6 at 45 °C for different time intervals (0 to 150 minutes). Then, aliquots were removed and the residual β-glucosidase activity was determined at 30 °C as described above. Two exponential models that describe the inactivation kinetics for one or two enzyme isoforms differing in heat stability were tested using iterative non-linear regression analysis (Motulsky & Christopoulos, 2004).

One phase decay: $$Y = (Y_0 - a) * \exp^{(-K * X)} + a \quad (4)$$

Two phase decay: $$Y = a + (Y_0 - a) * \text{Percent}_{Fast} * 0.01 * \exp^{(-KFast*X)} + (Y_0 - a) * (100 - \text{Percent}_{Fast}) * 0.01 * \exp^{(-KSlow*X)} \quad (5)$$

with Y_0 being the initial activity measured at $t = 0$ min, a being the residual activity at $t = 120$ min and K being the heat inactivation constant.

The half lives for the heat-labile and the heat-stable β-glucosidases were calculated as follows:

$$\text{Half life} = \frac{-\ln(\frac{1}{2})}{K}$$

The experimental data were fitted to equation (4) and (5) and parameters for heat inactivation were calculated with JMP 7.1 by performing an iterative non-linear regression analysis.

Statistical analysis

Before statistical analysis of the data, we tested the data for homogeneity of variance (Levene test) and normal distribution (Shapiro Wilk test). If necessary, data were log10-transformed to obtain homogeneity of variance and normal distribution. To compare the β-glucosidase activity of larvae reared on different host plants and to compare the β-glucosidase activity of the two lepidopteran species on the same host plant, we used the T-test for equal variances or the T-test for unequal variances respectively (Ruxton, 2006). Pearson correlations were calculated for larval weight as dependent variable and β-glucosidase activity as the independent variable.

To compare the fit between the kinetic models, we performed an F-test for nested models as proposed by Motulsky and Christopoulos (2004). As the null hypothesis, the simpler model including fewer parameters is assumed to be correct. The F-test to compare the model fit between nested models is calculated as indicated below, using the sum of squares and the degrees of freedom from the computed curve statistics that result from the iterative nonlinear regression analysis:

$$F = \frac{(SS_1 - SS_2)/(DF_1 - DF_2)}{SS_2/DF_2}$$

An F-ratio near 1.0 means that the simpler model is correct, while a much greater F-ratio with a P-value lower than 0.05 implies that the more complicated model provides a significantly better fit than the simple model (Motulsky & Christopoulos, 2004). All statistical tests were performed with JMP 7.1 (SAS) and Excel (Microsoft).

4.4 Results

Enzyme activity and kinetic properties of β-glucosidase

When plotting the β-glucosidase activity of *E. acraea* determined with NPβGlc against the pH, we found two maxima: one maximum at pH 6.3 and one maximum at pH 6.7. Between pH 6.0 and pH 7.0, the β-glucosidase activity of *E. acraea* larvae ranged between 90% and 100% of the maximum activity. The β-glucosidase of *J. coenia* larvae that was determined with NPβGlc had a bell-shaped activity profile with a maximum activity at pH 6.6. Here, the β-glucosidase retained between 85% and 100% of the maximum activity for pH-values ranging from 6.0 to 7.8.

We performed enzyme kinetic measurements for larvae of both lepidopteran species in the buffer in which we detected the maximum activity. For *J. coenia* larvae, the Lineweaver-Burk plot revealed that the kinetics of β-glucosidase in the midgut raw extract followed Michaelis-Menten behavior when using the synthetic substrate NPβGlc in the range from 0.4 mM to 5.6 mM (see larvae number 1 and 2, Table 4.1 and Figure 4.1 a). When we used substrate concentrations ranging from 0.4 mM to 32 mM, the Lineweaver-Burk plot clearly revealed substrate inhibition for the individuals number 3, 4, and 5 (Table 4.1, Figure 4.1 b).

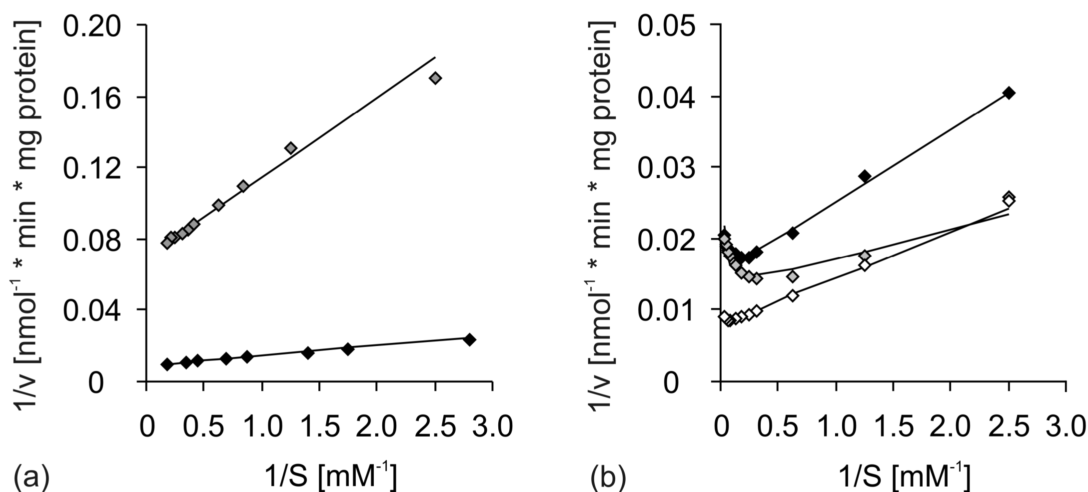

Figure 4.1 (a) and (b) Lineweaver-Burk plot of *Junonia coenia* β-glucosidase activity. The reciprocal β-glucosidase activity 1/v was plotted against the reciprocal substrate concentration 1/S. The points are experimental data and represent the means of two independent determinations for each substrate concentration. (a) The β-glucosidase activities of two larvae (black and grey diamonds) were determined with 0.4 - 5.6 mM 4-nitrophenyl-β-D-glucoside. The theoretical curves were derived from kinetic parameters determined by iterative nonlinear regression based on the simple Michaelis-Menten model. (b) The β-glucosidase activity of three larvae (black grey and white diamonds) reared on *Plantago lanceolata* was determined with 0 - 32 mM 4-nitrophenyl-β-D-glucoside. The theoretical curves were derived from kinetic parameters determined by iterative nonlinear regression based on the Michaelis-Menten model accounting for substrate inhibition.

Based on the graphical results of the Lineweaver-Burk plots, we compared the model for simple Michaelis-Menten kinetics (1) for the larvae number 1 and 2 where β-glucosidase activity was determined in the range of 0.4 mM to 5.6 mM with the model for substrate inhibition at higher substrate concentrations (3). For these two individuals, model (1) as the null hypothesis yielded a better result than the more complicated model (3) (Table 4.1). For the larvae number 3, 4, and 5 where β-glucosidase activity was determined in the range from 0.4 mM to 32 mM, model (1) was rejected in favor of model (3) with substrate inhibition ($p < 0.001$, for all three individuals; Table 4.1). For the substrate aucubin, the comparison of the fit for model (1) and (2) yielded a better fit for model (1) for all three individuals tested and resolved one β-glucosidase that followed typical Michaelis-Menten behavior. Kinetic parameters of *J. coenia* midgut β-glucosidases are given in Table 4.1.

When we plotted the β-glucosidase activity against substrate concentrations of NPβGlc ranging from 0 mM up to 80 mM, the observed kinetic of the β-glucosidase activity in the

midgut homogenates of the three tested *E. acraea* individuals followed Michaelis-Menten behavior (Figure 4.2a). We performed an iterative nonlinear regression analysis to obtain best fit values for V_{max} and K_m using equation (1) for one enzyme (β-glucosidase) and (2) for two enzymes (β-glucosidases). Then, the obtained curve fits of model (1) and (2) were compared using an F-test for nested models (see Material and Methods). The significant F-test confirmed that *E. acraea* larvae had two major midgut β-glucosidases with different Michaelis constants towards NPβGlc (Table 2). One β-glucosidase had a higher substrate affinity towards NPβGlc and the other β-glucosidase had a lower substrate affinity towards NPβGlc (Table 4.2). For the substrate aucubin, the Michaelis Menten plot indicated that β-glucosidase activity in the midgut homogenates of the three tested *E. acraea* individuals followed simple Michaelis-Menten behavior (Figure 4.2b). The additional statistical comparison of the fit for model (1) and (2) yielded a better fit for model (1) and resolved one β-glucosidase that followed typical Michaelis-Menten behavior.

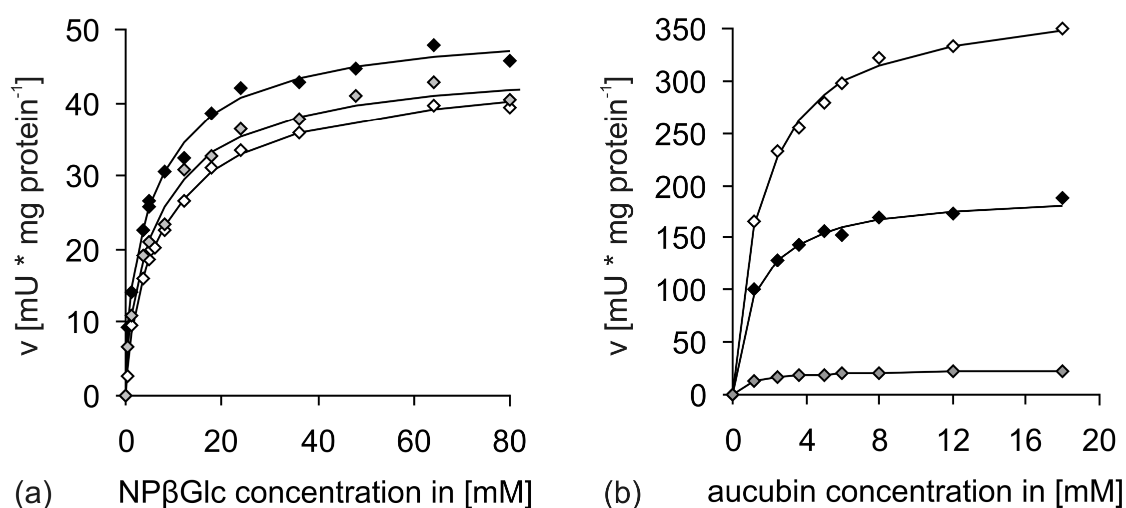

Figure 4.2 (a) and (b) Michaelis-Menten plots of *Estigmene acraea* β-glucosidase activity. The β-glucosidase activity was plotted against the used substrate concentration. The points are experimental data and represent the means of two independent determinations for each substrate concentration. (a) The β-glucosidase activities of three larvae (black, grey and white diamonds) reared on *Plantago major* were determined with 4-nitrophenyl-β-D-glucoside. The theoretical curves were derived from kinetic parameters determined by iterative nonlinear regression based on the Michaelis-Menten model implying the existence of two isoforms that differ in substrate affinity. (b) The β-glucosidase activities of three larvae (black, grey and white diamonds) reared on *P. lanceolata* were determined with the iridoid glycoside aucubin. The theoretical curves were derived from kinetic parameters that were determined by iterative non-linear regression based on the simple Michaelis-Menten model.

Table 4.1 Kinetic parameters (estimated means ± S.E.) for the substrates 4-nitrophenyl-β-D-glucoside (NPβGlc) and aucubin of *Junonia coenia* β-glucosidase of different individuals are given in this table. The kinetic parameters for NPβGlc for two individuals (number 1 was reared on *Plantago major,* and 2 was reared on *P. lanceolata*) were determined using substrate concentrations ranging from 0.4 to 5.6 mM, while for the other three individuals (number 3, 4, and 5 reared on *P. lanceolata*), NPβGlc concentrations were in the range from 0 mM to 32 mM. For the substrate aucubin, substrate concentrations ranged from 0 mM to 18 mM (n = 3, larvae were reared on *P. lanceolata*). Kinetic parameters were determined by iterative nonlinear regression based on the Michaelis-Menten-model (1) or the Michaelis-Menten-model accounting for substrate inhibition (3). P-values < 0.05 indicate that the more complex model (3) fits significantly better than the simpler model (1).

No	Host plant	Substrate	K_{m1} [mM]	V_{max1}		Substrate inhibition K_i [mM]	model	F-test	P-value
1	*P. major*	NPβGlc	0.64 ± 0.05	113.35 ± 2.53	[mU midgut^{-1}]	---	(1) vs. (3)	$F_{1,5}$ = 0.00	n.s.
2	*P. lanceolata*	NPβGlc	0.64 ± 0.03	14.30 ± 0.17	[mU midgut^{-1}	---	(1) vs. (3)	$F_{1,8}$ = 0.00	n.s.
3	*P. lanceolata*	NPβGlc	0.71 ± 0.19	68.73 ± 1.93	[mU mg protein^{-1}]	80.0 ± 12.6	(1) vs. (3)	$F_{1,8}$ = 64.88	P < 0.001
4	*P. lanceolata*	NPβGlc	0.34 ± 0.07	79.62 ± 3.82	[mU mg protein^{-1}]	43.9 ± 8.9	(1) vs. (3)	$F_{1,8}$ = 47.35	P < 0.001
5	*P. lanceolata*	NPβGlc	0.95 ± 0.08	134.25 ± 3.20	[mU mg protein^{-1}]	186.5 ± 45.2	(1) vs. (3)	$F_{1,8}$ = 21.98	P < 0.001

No	Aucubin	Substrate	K_{m1} [mM]	V_{max1} [mU * mg protein^{-1}]	K_{m2} [mM]	V_{max2} [mU * mg protein^{-1}]	model	F-test	P-value
6	*P. lanceolata*	Aucubin	1.85 ± 0.19	127.14 ± 3.39	---	---	(1) vs. (3)	$F_{2,5}$ = 0.02	n.s.
7	*P. lanceolata*	Aucubin	1.79 ± 0.16	164.95 ± 3.65	---	---	(1) vs. (3)	$F_{1,6}$ = 1.33	n.s.
8	*P. lanceolata*	Aucubin	1.50 ± 0.10	164.32 ± 2.52	---	---	(1) vs. (3)	$F_{1,6}$ = 0.00	n.s.

Table 4.2 Kinetic parameters (estimated means ± S.E.) of *Estigmene acraea* β-glucosidase are given in this table. The kinetic parameters for three different individuals reared on *Plantago major* were determined using concentrations of 4-nitrophenyl-β-D-glucoside (NPβGlc) ranging from 0 mM to 80 mM. For the substrate aucubin, three larvae were reared on *P. lanceolata* and kinetic parameters were determined for substrate concentrations ranging from 0 mM to 18 mM. Kinetic parameters were determined by iterative nonlinear regression based on the simple Michaelis-Menten-model (1) or the Michaelis-Menten-model including two isoforms with different Michaelis-constants and maximal activities (2). P-values < 0.05 indicate that the more complex model (2) fits significantly better than the simpler model (1).

No	Host plant	Substrate	K_{m1} [mM]	V_{max1} [mU * mg protein^{-1}]	K_{m2} [mM]	V_{max2} [mU * mg protein^{-1}]	model	F-test	P-value
1	*P. major*	NPβGlc	0.20 ± 0.24	11.14 ± 4.27	8.18 ± 2.03	39.66 ± 3.61	(1) vs. (2)	$F_{2,10} = 18.89$	$P < 0.001$
2	*P. major*	NPβGlc	0.13 ± 0.39	6.41 ± 4.50	8.01 ± 2.16	38.83 ± 3.93	(1) vs. (2)	$F_{2,10} = 6.37$	$P < 0.05$
3	*P. major*	NPβGlc	1.03 ± 0.87	9.32 ± 6.04	11.00 ± 3.14	35.11 ± 5.33	(1) vs. (2)	$F_{2,10} = 9.23$	$P < 0.02$

No	Aucubin	Substrate	K_{m1} [mM]	V_{max1} [mU * mg protein^{-1}]	K_{m2} [mM]	V_{max2} [mU * mg protein^{-1}]	model	F-test	P-value
1	*P. lanceolata*	Aucubin	1.20 ± 0.12	192.96 ± 4.06	---	---	(1) vs. (2)	$F_{2,5} = 4.07$	n.s.
2	*P. lanceolata*	Aucubin	1.12 ± 0.12	23.82 ± 0.50	---	---	(1) vs. (2)	$F_{2,5} = 0.92$	n.s.
3	*P. lanceolata*	Aucubin	1.60 ± 0.09	378.71 ± 5.25	---	---	(1) vs. (2)	$F_{2,5} = 1.16$	n.s.

Heat inactivation of J. coenia midgut β-glucosidase activity

We determined the residual β-glucosidase activity after incubating β-glucosidase extracts of three *J. coenia* caterpillars for different time frames at 45 °C. The remaining relative activity was plotted against the time. The inactivation curves show a biphasic decrease of midgut β-glucosidase for *J. coenia* (Figure 4.3).

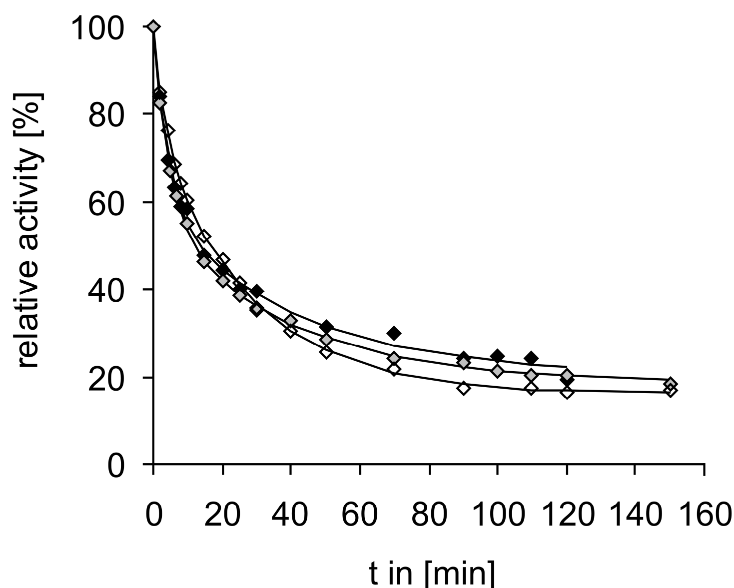

Figure 4.3 Thermal inactivation of midgut β-glucosidases activity of *Junonia coenia* at 45°C. The residual β-glucosidase activities of three larvae (black diamonds, grey squares and white triangles) after thermal inactivation were determined with 5.7 mM 4-nitrophenyl-β-D-glucoside (NPβGlc) in 100 mM citrate sodium phosphate buffer (pH 6.6). The points are experimental data and represent the means of two independent determinations for each time point. The theoretical curves were derived from parameters determined by iterative nonlinear regression based on the two phase decay model. Parameters are listed in Table 4.3.

Comparing the fit of model (4) and (5) to the data, the model of one phase exponential decay (corresponding to one β-glucosidase) was rejected in favor of the two phase exponential decay model that describes the exponential decay velocity of two enzymatic isoforms with different heat stability. Model (5) provided a better fit for all three individuals tested (Table 4.3). 50% versus 25% of the entire β-glucosidase activity measured with NPβGlc could be attributed to a more heat-labile isoform, whereas 75% versus 50% corresponded to a β-glucosidase with higher heat stability. The respective heat inactivation constants are given in Table 4.3.

Table 4.3 Heat inactivation constants of *Junonia coenia* β-glucosidases were determined for three different larvae reared on *Plantago lanceolata*. Based on equation (5) which describes the two phase decay of the β-glucosidase inactivation, the heat inactivation parameters were determined by iterative nonlinear regression and represent the estimated mean ± standard error, with a being the residual β-glucosidase activity [U mg protein^{-1}] where the inactivation curve plateaus at some value (zero or other than zero), $Percent_{Fast}$ representing the percentage of the heat-labile β-glucosidase fraction in the midgut, K_{Fast} being the heat inactivation constant for the heat-labile, and K_{Slow} being the heat inactivation constant for the heat-stable β-glucosidase. The half lives of the heat-labile and the heat-stable β-glucosidase in the midgut were calculated based on the inactivation constants K_{Fast} and K_{Slow} respectively (-ln (0.5)/K). P-values < 0.05 indicate that the more complex two-phase decay model (5) fits significantly better than the simpler model for one-phase decay (4).

Parameter	Jcoe_1	Jcoe_2	Jcoe_3
Y0 =100 %	99.9 ± 1.8	99.5 ± 0.7	100.0 ± 0.7
Plateau a in %	20.3 ± 2.5	18.4 ± 0.7	16.1 ± 0.4
$Percent_{Fast}$ in [%]	50.7 ± 5.5	52.3 ± 2.6	24.7 ± 1.9
K_{Fast} [min^{-1}]	0.233 ± 0.045	0.204 ± 0.016	0.351 ± 0.047
K_{Slow} [min^{-1}]	0.025 ± 0.007	0.026 ± 0.003	0.037 ± 0.001
Half $life_{Fast}$ [min] = $-\ln(0.5)/K_{Fast}$	3.0 ± 0.6	3.4 ± 0.3	2.0 ± 0.3
Half $life_{Slow}$ [min] = $-\ln(0.5)/K_{Slow}$	27.9 ± 7.7	26.8 ± 2.8	18.6 ± 0.7
F-test	$F_{2,12} = 34.21$	$F_{2,12} = 186.98$	$F_{2,12} = 136.58$
P-value	P < 0.001	P < 0.001	P < 0.001

After thermal inactivation at 45 °C, the residual β-glucosidase activity was determined in 100 mM citrate-sodium phosphate buffer (pH 6.6) with 5.7 mM 4-nitrophenyl-β-D-glucoside as substrate.

The β-glucosidase activity in response to the host plants

To test whether the different iridoid glycoside composition and concentration in the host plants differentially influenced β-glucosidase activity, we compared the specific midgut β-glucosidase activity per mg protein of *J. coenia* and *E. acraea* larvae reared on the two *Plantago* species. *J. coenia* larvae on *P. major* had a significantly lower β-glucosidase activity

towards NPβGlc than larvae reared on *P. lanceolata* (T-test, $t = -2.164$, $df = 13$, $p < 0.050$; Figure 4.4a). Using the iridoid glycoside aucubin as substrate, we detected a very low activity in the larvae reared on *P. major*, but found a significantly higher β-glucosidase activity in the larvae reared on *P. lanceolata* (T-test for unequal variances, $t = -4.736$, $df = 6.28$, $p < 0.003$; Figure 4.4a).

The β-glucosidase activity towards aucubin was not significantly correlated with the β-glucosidase activity towards NPβGlc ($r^2 = 0.503$, $F_{1,5} = 4.068$, $p = 0.114$). When we tested for interaction of β-glucosidase activity and larval weight in *J. coenia* on *P. lanceolata*, we found that larval weight was not correlated with either of the β-glucosidase substrates ($r^2_{NP\beta Glc} = 0.079$, $F_{1,9} = 0.683$, $p = 0.433$, $r^2_{aucubin} = 0.283$, $F_{1,5} = 1.579$, $p = 0.277$).

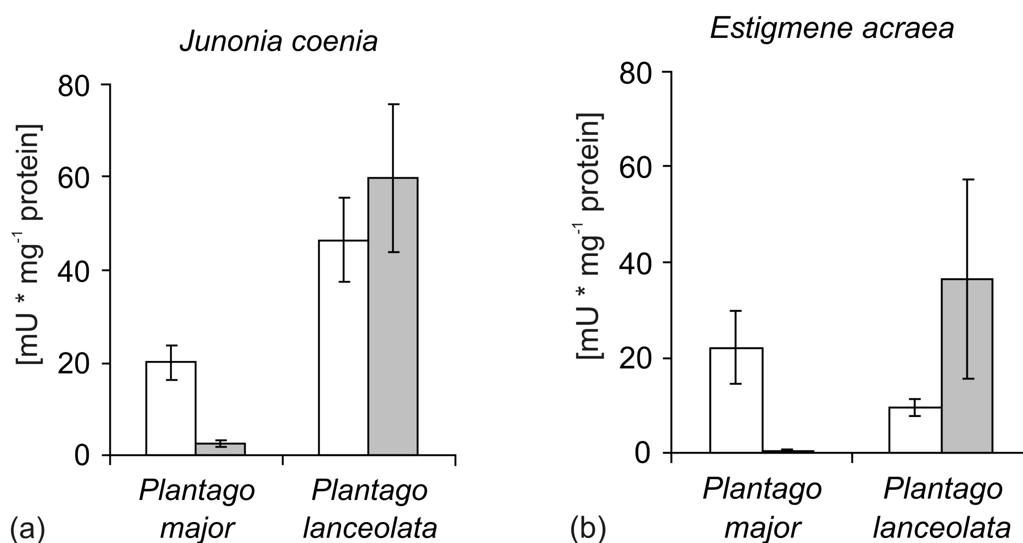

Figure 4.4 (a) and (b) The β-glucosidase activity of *Junonia coenia* and *Estigmene acraea* in response to two host plants, *Plantago major* (low iridoid glycosides) or *P. lanceolata* (high iridoid glycosides). The enzymatic activities (means ± S.E.) were determined with 5 mM 4-nitrophenyl-β-D-glucose (white bars) and 5 mM aucubin (grey bars). (a) β-Glucosidase activity of *J. coenia* last instar larvae on *P. major* ($n_{NP\beta Glc} = 5$, $n_{aucubin} = 5$) and on *P. lanceolata* ($n_{NP\beta Glc} = 10$, $n_{aucubin} = 6$). (b) β-Glucosidase activity of *E. acraea* last instar larvae reared on *P. major* ($n_{NP\beta Glc} = 10$, $n_{aucubin} = 5$) or on *P. lanceolata* ($n_{NP\beta Glc} = 10$, $n_{aucubin} = 10$)

When comparing the midgut β-glucosidase activity of *E. acraea* larvae reared on the two *Plantago* species, we found that the β-glucosidase activity towards NPβGlc was significantly lower in the larvae reared on *P. lanceolata*, the plant species with the higher iridoid glycoside content (T-test, $t = 2.233$, $df = 18$, $p < 0.05$; Figure 4.4b). Using the iridoid glycoside aucubin as substrate, we detected a very low β-glucosidase activity in the larvae reared on *P. major*.

However, in the larvae reared on *P. lanceolata*, we found a significantly higher aucubin hydrolyzing β-glucosidase (T-test for unequal variances, $t = -3.133$, $df = 9.82$, $p < 0.011$; Figure 4.4b). The β-glucosidase activity towards aucubin was significantly negatively correlated with the β-glucosidase activity towards NPβGlc when the larvae were reared on *P. major* ($r^2 = 0.813$, $F_{1,4} = 13.071$, $p < 0.036$). However, we did not find a significant correlation between larval weight and β-glucosidase activity towards either of the substrates. In contrast, in the larvae reared on *P. lanceolata*, the β-glucosidase activity towards aucubin was significantly positively correlated with the β-glucosidase activity towards NPβGlc ($r^2 = 0.470$, $F_{1,9} = 7.102$, $p < 0.029$). In addition, the β-glucosidase activity towards aucubin was significantly positively correlated with larval weight when the larvae were reared on *P. lanceolata* ($r^2 = 0.492$, $F_{1,9} = 7.737$, $p < 0.024$), whereas the β-glucosidase activity towards NPβGlc of the same larvae was not correlated with larval weight ($r^2 = 0.031$, $F_{1,9} = 0.256$, $p = 0.626$).

To test for differences between the specialist and the generalist to varying iridoid glycoside levels in *Plantago*, we compared the β-glucosidase activity of the specialist with that of the generalist. While the β-glucosidase activity measured with NPβGlc did not differ between the two lepidopteran species reared on *P. major* (T-test, $t = 0.339$, $df = 11$, $p = 0.741$), the larvae of the specialist *J. coenia* had a significantly higher β-glucosidase activity towards NPβGlc than the generalist *E. acraea* when the larvae were reared on *P. lanceolata* (T-test, $t = 5.728$, $df = 18$, $p < 0.0001$). When reared on *P. major*, the larvae of both species had a very low β-glucosidase activity towards aucubin that was, however, significantly higher for *J. coenia* (T-test, $t = 4.017$, $df = 8$, $p < 0.004$). When reared on *P. lanceolata*, the β-glucosidase activity towards aucubin did not differ between the two species (T-test, $t = 1.793$, $df = 14$, $p = 0.095$).

4.5 Discussion

In this study, we examined whether midgut β-glucosidase might play a crucial role for generalist and specialist herbivores in adaptation to iridoid glycoside containing host plants. For that, we performed enzyme kinetic experiments to compare substrate affinities between the generalist and specialist. Both lepidopteran species have two midgut β-glucosidases that are active against the standard β-glucosidase substrate NPβGlc. NPβGlc is cleaved by class B β-glucosidases that are also active against plant glycosides (Terra & Ferreira, 2005). The Michaelis-Menten kinetic using NPβGlc as substrate revealed that the β-glucosidase of

the specialist *J. coenia* is inhibited by substrate concentrations higher than 5.6 mM (Table 4.1). This phenomenon has been shown for other insect glycosidases (Azevedo et al., 2003, Genta et al., 2007, Pankoke et al., submitted). However in some cases, the observed pattern of substrate inhibition in β-glucosidases is an artifact. In these cases, it results from transglycosylation caused by the reaction of enzyme products with substrate molecules (Cristofoletti et al., 2003, Ferreira et al., 2001). We did not check for glucosyl derivatives indicating that transglycosylation has occurred and therefore, we can not say for certain whether the observed pattern should be attributed to substrate inhibition or to transglycosylation. In contrast to *J. coenia*, the generalist *E. acraea* had two midgut β-glucosidases, one β-glucosidase with high and one with low substrate affinity (Table 4.2). The Michaelis constants of the β-glucosidase of *J. coenia* and that of the high affinity β-glucosidase of *E. acraea* are very similar to other biochemically characterized and purified lepidopteran β-glucosidases (Azevedo et al. 2003; Marana et al. 2000; Pratviel-Sosa et al. 1987; Santos and Terra 1985). The second β-glucosidase with a much lower affinity towards NPβGlc that we found in *E. acraea* resembles the one found in another arctiid, *Grammia incorrupta* (Pankoke et al., in preparation). So far, Michaelis constants indicating such a reduced substrate affinity for 4-(α- or β-)nitrophenyl-glucose substrates have been reported for insect glucosidases only rarely (Baker, 1991, Baker & Woo, 1992, Terra & Jordão, 1989).

As a second β-glucosidase substrate, we used the iridoid glycoside aucubin that occurs in both *Plantago* species in different concentrations (Barton & Bowers, 2006, Fontana et al., 2009, Marak et al., 2000, Rønsted et al., 2003, Willinger & Dobler, 2001, Wurst & Van Der Putten, 2007). Only when the larvae of *J. coenia* and *E. acraea* were reared on the high iridoid glycoside plant *P. lanceolata*, did we find, for both caterpillar species, substantial amounts of β-glucosidase that were able to hydrolyze aucubin. Thus, we used individuals reared on *P. lanceolata* to determine Michaelis constants. Most interestingly, the Michaelis constants for aucubin of the iridoid glycoside specialist *J. coenia* and the generalist *E. acraea* were very similar (Table 4.1 and Table 4.2). We found an aucubin hydrolyzing β-glucosidase in *G. incorrupta* (see chapter 3) that also had a similar Michaelis constant close to that of *J. coenia*. For the specialist and these two generalist species, the substrate affinity towards aucubin was substantially higher than that of two *Papilio* species differentially adapted towards phenolic glycosides (Lindroth, 1988). To protect the herbivores from autotoxicity when feeding on diets with high plant glycoside concentrations, it was proposed that herbivores reduce their β-glucosidase activity and evolve β-glucosidases with lower Michaelis

constants in response to the respective glycosides (Ferreira et al., 1997, Marana et al., 2000). With respect to the very similar Michaelis constants of the generalists and the iridoid glycoside specialist, we can not confirm the hypothesis that specialists necessarily should evolve β-glucosidases with reduced substrate affinities towards toxic plant glycosides. This is at least not the case for the iridoid glycoside specialist *J. coenia*.

By performing heat inactivation experiments, we found two β-glucosidases for the iridoid glycoside specialist *J. coenia* reared on *P. lanceolata*. They could not be differentiated by performing Michaelis-Menten kinetics due to substrate inhibition or transglycosylation reactions at higher substrate concentrations (see above). For two individuals, we found that the two β-glucosidases were present in equal proportions (heat-labile versus heat-stable β-glucosidase). These two individuals also showed very similar heat inactivation constants and half lives for their isoenzymes. The third individual had 25% of the heat-labile and 75% of heat-stable β-glucosidase, and their respective heat inactivation constants were higher than those of the β-glucosidases determined for two other *J. coenia* individuals. Half lives of these two enzymes were shorter as well. Compared to other lepidopteran β-glucosidases, the heat inactivation rate constants of the two β-glucosidases of *J. coenia* are similar to the inactivation rate constants of the two β-glucosidases of the arctiid *S. virginica* (see chapter 2). The heat inactivation rate constant of the heat-labile β-glucosidase of *J. coenia* is between three fold and six fold higher than that of *Erinnyis ello*'s midgut β-glucosidase (10×10^{-4} sec), while the heat inactivation rate constant of the more heat-stable β-glucosidase of *J. coenia* is half of that from *E. ello* β-glucosidase (Santos & Terra, 1985).

When the larvae in our feeding experiment were reared on *P. major*, the generalist and the specialist did not significantly differ in β-glucosidase activity for the substrate NPβGlc. When we used aucubin as substrate, the β-glucosidase activity determined for both species was very close to the limit of detection of the spectrophotometer. Although we found a significantly higher activity towards aucubin in the specialist, these results have to be interpreted with caution. Feeding on *P. lanceolata* had significantly different effects on the β-glucosidase activity determined with NPβGlc in the generalist compared to the specialist. The generalist *E. acraea* reduced, while the specialist *J. coenia* increased, its total β-glucosidase activity significantly. The observed differences in β-glucosidase activity between the generalist and the specialist were most likely influenced by iridoid glycoside concentrations present in the *Plantago* species used in our experiment. While the amount of aucubin in *P. major* ranges

from 0.07% to 1% of the plant dry weight (Barton & Bowers, 2006, Rønsted et al., 2003, Willinger & Dobler, 2001), the leaves of *P. lanceolata* have a total iridoid glycoside concentration ranging from 1% -12 % of the plant dry weight with a catalpol:aucubin ratio of usually 0.5 to 0.7 (Fontana et al., 2009, Marak et al., 2000, Willinger & Dobler, 2001, Wurst & Van Der Putten, 2007). For two other arctiids, the β-glucosidase activity measured with NPβGlc was likewise negatively correlated with increasing iridoid glycoside concentrations in the host plants (see chapter 2 and chapter 3). In general, the decrease of β-glucosidase has been shown as potential mechanism to be able to tolerate toxic glycosides in the diet (Desroches et al., 1997, Ferreira et al., 1997, Lindroth, 1988, Mainguet et al., 2000). A decrease of β-glucosidase might help to keep the level of the detrimental secondary plant aglycones low which, in turn, could help to reduce or avoid autotoxicity. This matches the "toxin dilution hypothesis" (Bernays et al., 1994, Freeland & Janzen, 1974, Hägele & Rowell-Rahier, 1999, Marsh et al., 2006, Singer et al., 2002). In comparison, the iridoid glycoside specialist *J. coenia* maintains a high β-glucosidase activity on *P. lanceolata*. This indicates that *J. coenia* has a different regulation mechanism from the arctiid generalist *E. acraea* in response to high iridoid glycosides in the diet.

In the larvae of both *J. coenia* and *E. acraea,* we found a significant induction of an aucubin hydrolyzing β-glucosidase when reared on *P. lanceolata*, the plant with the higher iridoid glycoside content. The hydrolysis of aucubin was shown to denature and cross-link proteins by alkylating amino acids with nucleophilic groups (e.g. –SH and $–NH_2$) (Konno et al., 1999). Furthermore, amino acids with nucleophilic groups (like lysine, histidine cysteine and methionine) are nutritionally limiting to insects (Felton et al., 1992). For the generalist *E. acraea*, the increase of the aucubin hydrolyzing β-glucosidase contrasts with the decrease of β-glucosidase activity when using NPβGlc as substrate. A similar induction of an aucubin hydrolyzing β-glucosidase feeding on *P. lanceolata* was shown for the arctiid *G. incorrupta* (see chapter 3). For *G. incorrupta* larvae, feeding on *P. lanceolata* led to a significantly reduced larval performance that was most likely caused by the β-glucosidase activity towards aucubin (see chapter 3). In comparison, the mean β-glucosidase activity towards aucubin in *E. acraea* larvae is almost four fold higher than in *G. incorrupta* larvae feeding on *P. lanceolata.* And rather surprisingly, the β-glucosidase activity towards aucubin is positively correlated with larval weight in *E. acraea*. As we did not determine larval performance on the two *Plantago* species, these results are difficult to interpret. For the specialist *J. coenia*, we could not detect a correlation between larval weight and the

β-glucosidase activity towards either of the substrates. Obviously, the feeding of *J. coenia* larvae on *P. lanceolata* goes together with a high β-glucosidase activity towards aucubin. Although this might lead to reduced protein availability, larval development is short and takes as little as three weeks (personal observation). So, potentially *J. coenia* larvae on *P. lanceolata* do profit from the high β-glucosidase activity towards aucubin. However, the question arises of how detoxification of the released iridoid aglycones might take place. Especially, because Adler et. al. (1995) could show in a previous study that the survival of *J. coenia* larvae feeding on *P. lanceolata* genotypes with high aucubin concentrations was significantly lower than on genotypes with low aucubin concentrations. So, the high β-glucosidase activity towards aucubin might have negative fitness costs when larvae feed on plant genotypes with high iridoid glycoside concentrations leading to an enhanced larval mortality.

Taken together, the polyphagous arctiid *E. acraea* and the oligophagous iridoid glycoside specialist *J. coenia* do not differ as much as predicted by their different feeding strategies. The generalist, on the one hand, shows a decrease of β-glucosidase activity also reported for two other arctiids (see chapter 2 and 3). On the other hand, *E. acraea* does not differ from the specialist in its induction of an aucubin hydrolyzing β-glucosidase when feeding on *P. lanceolata*. Even the Michaelis constants for aucubin are similar. So it seems likely that both the polyphagous generalist *E. acraea* and the specialist *J. coenia* must have another mechanism (or mechanisms) for tolerating iridoid glycosides in their diet, instead of solely relying on β-glucosidase as crucial key enzyme. A potential mechanism to adapt to strongly alkylating plant compounds like iridoid glycosides was shown to be the active secretion of glycine into the midgut (Konno et al., 1996, Konno et al., 1997, Konno et al., 2001, Konno et al., 1998), where the glycine counteracts the protein denaturing activities of an iridoid (Konno et al., 1998). The secretion of glycine into the midgut has developed independently in several species from different lepidopteran families that feed on *Ligustrum*. Thus, Konno et al. (1998) proposed the secretion of glycine as potential mechanism for other herbivores feeding on plants with protein denaturing activities. It is possible that both, the generalist *E. acraea* as well as the specialist *J. coenia*, might have developed a similar mechanism to counteract the protein-denaturing activity of iridoid glycosides. Then, the β-glucosidase activity towards aucubin would not present an obstacle, but would provide substantial amounts of additional glucose available to the insect.

5 Abschließende Diskussion

5.1 Einfluss von iridoidglycosidhaltigen Wirtspflanzen auf die β-Glucosidase-Aktivität von *Spilosoma virginica*

Im ersten Kapitel meiner Dissertation habe ich den Einfluss von iridoidglycosidhaltigen Wirtspflanzen auf die β-Glucosidase-Aktivität des nordamerikanischen Nahrungsgeneralisten *Spilosoma virginica* (Arctiidae) untersucht. Mit Hitzeinaktivierungsversuchen konnte ich für *S. virginica* Raupen zeigen, dass diese zwei β-Glucosidase-Isoformen besaßen. Durch eine Bestimmung der β-Glucosidase-Aktivität bei unterschiedlichen Substratkonzentrationen ergab sich, dass die beiden β-Glucosidase-Isoformen sich allerdings nicht anhand ihrer Substrataffinität voneinander unterscheiden lassen. Die Michaelis-Menten-Konstante für die β-Glucosidase-Isoformen von *S. virginica* ist hierbei vergleichbar mit den Michaelis-Menten-Konstanten verschiedener Lepidopterenarten, deren β-Glucosidasen biochemisch charakterisiert worden sind (Azevedo et al., 2003, Marana et al., 2000, Pratviel-Sosa et al., 1987, Santos & Terra, 1985). Bei *S. virginica* nahm die Gesamt-β-Glucosidase-Aktivität bei höheren Konzentrationen des Substrats 4-Nitrophenyl-β-D-Glucose ab. Dieses Phänomen lässt sich entweder auf Substratinhibition oder Transglycosylierung zurückführen, da sich bei beiden die Enzymaktivität bei höheren Substratkonzentrationen verringert (Azevedo et al., 2003, Ferreira et al., 2001). Durch Transglycosylierung entstehen außerdem Substratderivate von 4-Nitrophenyl-β-D-Glucose, da durch β-Glucosidase weitere Glucosemoleküle an das Substrat gebunden werden. Für *S. virginica* kann ich keine definitive Aussage dazu machen, ob nun Substratinhibition oder Transglycosylierung zu der beobachteten Verminderung von β-Glucosidase-Aktivität bei hohen Substratkonzentrationen geführt hat. Dies ist jedoch auch nicht relevant für die Anpassung an Iridoidglycoside in der Nahrung.

Um den Einfluss verschiedener Wirtspflanzen auf die β-Glucosidase-Aktivität zu untersuchen, wurden die Raupen von *S. virginica* im fünften Larvalstadium vor der Präparation für zwei Wochen auf Breitwegerich (*P. major*) oder Spitzwegerich (*P. lanceolata*) umgesetzt oder weiter auf Löwenzahn (*T. officinale*) belassen. Auf die unterschiedlichen Iridoidglycosidkonzentrationen in den Wirtspflanzen reagierten die Raupen von *S. virginica* mit einer Reduzierung der mit dem Substrat 4-Nitrophenyl-β-D-Glucose nachgewiesenen Gesamt-β-Glucosidase-Aktivität. Hierbei war die Aktivität der β-Glucosidase im Mittel umso geringer, je höher die Konzentration der Iridoidglycoside in

den Wirtspflanzen war. Ich untersuchte weiterhin, ob die β-Glucosidase von *S. virginica* Iridoidglycoside hydrolysieren würde. Interessanterweise fand sich nur eine geringfügig nachweisbare β-Glucosidase-Aktivität für das Iridoidglycosid Aucubin, die sich nicht signifikant zwischen den Wirtspflanzen unterschied. Insgesamt deuten die Ergebnisse darauf hin, dass es sich bei der Reduzierung der β-Glucosidase-Aktivität um eine adaptive Reaktion auf Sekundärstoffe in den Wirtspflanzen handelt. Die Reduzierung von β-Glucosidase-Aktivität konnte auch schon bei anderen Insektenarten beobachtet werden (Desroches et al., 1997, Ferreira et al., 1997, Lindroth, 1988, Mainguet et al., 2000), wobei die Regulation offensichtlich eine relevante Anpassung an steigende Glycosidkonzentrationen in der Nahrung darstellt.

5.2 β-Glucosidase hat negative Auswirkungen auf die larvale „Performance“ von *Grammia incorrupta* auf iridoidglycosidhaltigen Wirtspflanzen

Die β-Glucosidase-Aktivität gegenüber Iridoidglycosiden hat zur Folge, dass die freigesetzten Aglykone kovalent an Amino- und Schwefelgruppen von Proteinen binden und dadurch die Proteinverfügbarkeit und -qualität verringern (Bartholomaeus & Ahokas, 1995, Kim et al., 2000, Konno et al., 1999). Die Proteinverfügbarkeit ist von essentieller Bedeutung für das Larvalwachstum (Felton, 1996, Felton et al., 1992, Lee et al., 2002). Aus diesem Grund habe ich in Kapitel 2 die Auswirkung von zwei Wirtspflanzen auf die Larvalentwicklung des Nahrungsgeneralisten *Grammia incorrupta* (Arctiidae) untersucht, wobei die Raupen die gesamte Larvalzeit entweder auf Löwenzahn (*T. officinale*) oder Spitzwegerich (*P. lanceolata*) gehalten wurden. Tatsächlich war die Larvalentwicklung signifikant länger, wenn *P. lanceolata* als Futterpflanze der Raupen diente. Als nächstes wurde untersucht, ob es einen Zusammenhang zwischen der verlängerten Larvalentwicklung auf *P. lanceolata* und der β-Glucosidase-Aktivität gibt. Die mit 4-Nitrophenyl-β-D-Glucose bestimmten β-Glucosidase-Aktivität der Raupen auf den beiden Wirtspflanzen unterschied sich signifikant, wobei die β-Glucosidase-Aktivität der Raupen auf *P. lanceolata* im Vergleich zu der auf *T. officinale* wieder reduziert war. Auch für diesen polyphagen Generalisten scheint die Reduzierung der mit 4-Nitrophenyl-β-D-Glucose bestimmten Gesamt-β-Glucosidase-Aktivität offensichtlich eine adaptive Reaktion auf die hohen Iridoidglycosidkonzentrationen in *P. lanceolata* zu sein. Um zu testen ob es tatsächlich einen Zusammenhang zwischen Larvalgewicht und β-Glucosidase-Aktivität auf den beiden Wirtspflanzen gab, bezog ich das Larvalgewicht als Kovariate mit in die statistische Untersuchung ein. Für die Raupen auf *T. officinale* waren

Larvalgewicht und β-Glucosidase-Aktivität positiv korreliert, während das Larvalgewicht und die β-Glucosidase-Aktivität der Raupen auf *P. lanceolata* negativ korrelierten. Das Ergebnis dieser statistischen Analyse unterstützt die Hypothese, dass β-Glucosidase einen signifikanten Einfluss auf die Larvalentwicklung hat. Offensichtlich führt die Fähigkeit der Raupen auf *P. lanceolata*, die mit 4-Nitrophenyl-β-D-Glucose messbare β-Glucosidase-Aktivität zu reduzieren dazu, dass sie ein höheres Gewicht erreichen und sich schneller entwickeln als diejenigen, die nicht über diese Eigenschaft verfügen. Eine verlängerte Larvalentwicklung könnte im Freiland zu einem höheren Risiko der Raupen führen, parasitiert oder gefressen zu werden (Singer, 2001). Das Larvalgewicht ist außerdem relevant für die Fitness, da es mit der Fertilität und damit mit der Anzahl an potentiellen Nachkommen eines Organismus korreliert.

Hinsichtlich der β-Glucosidase-Aktivität, die mit dem Iridoidglycosid Aucubin als Substrat bestimmt wurde, fand ich ebenfalls einen signifikanten Einfluss der beiden Wirtspflanzenarten. Während die Raupen auf *T. officinale* nur eine sehr geringe Aktivität gegenüber Aucubin aufwiesen, war in den Raupen auf *P. lanceolata* die β-Glucosidase-Aktivität signifikant erhöht. *P. lanceolata* enthält bis zu 12% des Trockengewichts an Iridoidglycosiden, wobei der Anteil an Aucubin im Vergleich zu Catalpol meistens gleich oder höher ist (Adler et al., 1995, Barton & Bowers, 2006, Fontana et al., 2009, Fuchs & Bowers, 2004, Klockars et al., 1993, Marak et al., 2000, Willinger & Dobler, 2001, Wurst & Van Der Putten, 2007). Die Spaltung von Aucubin durch β-Glucosidase in den Raupen, die auf *P. lanceolata* gehalten wurden, sollte also zu einer deutlich verringerten Proteinverfügbarkeit führen und diese wiederum zu verringerten Wachstumsraten der Raupen. Meine Ergebnisse belegen dies in zweierlei Hinsicht. Im Vergleich zu der Wirtspflanzenart *T. officinale* (ohne Iridoidglycoside) war die Gewichtszunahme der Raupen auf der Wirtspflanzenart *P. lanceolata*, die Iridoidglycoside enthält, signifikant niedriger. Des Weiteren waren Larvalgewicht und β-Glucosidase-Aktivität gegenüber Aucubin negativ korreliert, das heißt, Raupen konnten nur dann ein höheres Gewicht erreichen, wenn sie eine geringere β-Glucosidase-Aktivität gegenüber Aucubin aufwiesen. Beide Ergebnisse sprechen dafür, dass die deutlich längere Larvalphase bei den Raupen von *G. incorrupta* auf *P. lanceolata* durch die β-Glucosidase-Aktivität gegenüber Iridoidglycosiden erklärt werden kann.

Trotzdem gibt es einen Widerspruch: Weshalb reduzieren die Larven auf *P. lanceolata* die β-Glucosidase-Aktivität gegenüber 4-Nitrophenyl-β-D-Glucose, was zu einem höheren

Larvalgewicht führt, wenn es gleichzeitig in den Larven eine β-Glucosidase gibt, die Aucubin umsetzt und damit die Larvalentwicklung deutlich verlangsamt? Um diese Frage besser beantworten zu können, habe ich untersucht, ob die β-Glucosidase-Aktivität, die ich mit den beiden Substraten 4-Nitrophenyl-β-D-Glucose und Aucubin bestimmt habe, für die Raupen auf den beiden Wirtpflanzen miteinander korreliert. Interessanterweise gab es keine Korrelation zwischen der β-Glucosidase-Aktivität für die beiden Substrate 4-Nitrophenyl-β-D-Glucose und Aucubin. Dies spricht dafür, dass die β-Glucosidase-Aktivität gegenüber Aucubin möglicherweise nicht tierischen Ursprungs ist, sondern beispielsweise von Mikroorganismen der Darmflora synthetisiert werden könnte. Dies würde erklären, weshalb die adaptive Reduktion von β-Glucosidase offensichtlich bei den meisten Raupen nicht ausreicht, um den schädlichen Einfluss der Iridoidglycoside auf das Larvalwachstum zu unterbinden.

5.3 Die β-Glucosidasen des Nahrungsgeneralisten *Estigmene acraea* und des Iridoidglycosidspezialisten *Junonia coenia* im Vergleich

Im dritten Kapitel verglich ich den Nahrungsgeneralisten *Estigmene acraea* (Arctiidae) mit dem Iridoidglycosidspezialisten *Junonia coenia* (Nymphalidae), wobei ich wieder die Auswirkungen von unterschiedlichen Iridoidglycosidkonzentrationen zweier iridoidglycosidhaltiger Wirtspflanzenarten auf die β-Glucosidase-Aktivität der Larven untersuchte. Beide Arten verfügten über β-Glucosidase-Aktivität, die das Substrat 4-Nitrophenyl-β-D-Glucose umsetzte. Die Untersuchungen zur Enzymkinetik ergaben, dass der Generalisten *E. acraea* zwei β-Glucosidase-Isoformen aufwies, die sich deutlich in ihrer Substrataffinität unterschieden. Für den Iridoidglycosidspezialisten *J. coenia* zeigten Hitzeinaktivierungsversuche, dass die Raupen ebenfalls zwei β-Glucosidase-Isoformen besaßen, die sich allerdings nicht hinsichtlich ihrer Substrataffinität unterschieden. Höhere Substratkonzentrationen führten vielmehr zu einer verringerten Enzymaktivität, die wieder auf Substratinhibition oder auf Transglycosylierung (siehe oben) zurückzuführen sein könnte (Azevedo et al., 2003, Ferreira et al., 2001).

Die beiden iridoidglycosidhaltigen Wirtspflanzen *P. major* und *P. lanceolata* hatten ebenfalls deutliche Effekte auf die β-Glucosidase-Aktivität der beiden Lepidopterenarten. Wurden die Larven des Generalisten und des Spezialisten auf *P. major* gehalten, dann unterschied sich die mit 4-Nitrophenyl-β-D-Glucose bestimmte β-Glucosidase-Aktivität nicht signifikant zwischen

den beiden Arten. Wurde die β-Glucosidase-Aktivität hingegen mit Aucubin als Substrat bestimmt, war diese für beide Schmetterlingsarten auf *P. major* sehr gering und lag nah an der Nachweisgrenze des Photometers. Aus diesem Grund sollte die trotzdem signifikant höhere β-Glucosidase-Aktivität des Spezialisten auch mit Vorsicht interpretiert werden.

Für die Raupen des Generalisten und des Spezialisten, die auf *P. lanceolata* gehalten wurden, ergab sich ein anderes Bild. Der Generalist reduzierte die mit 4-Nitrophenyl-β-D-Glucose bestimmte β-Glucosidase-Aktivität, während der Spezialist die β-Glucosidase-Aktivität induzierte. Beide Arten, *J. coenia* und *E. acraea*, induzierten ähnlich wie die Larven von *G. incorrupta* eine Aucubin hydrolysierende β-Glucosidase, wobei die gemessene Aktivität des Iridoidglycosidspezialisten signifikant höher als die β-Glucosidase-Aktivität des Generalisten war. Hierbei zeigte die Bestimmung der kinetischen Enzymeigenschaften, dass die Aucubin hydrolysierende β-Glucosidase des Nahrungsgeneralisten *E. acraea* eine ähnlich hohe Substrataffinität zu Aucubin aufwies wie die des Iridoidglycosidspezialisten *J. coenia*. Weitere Analysen ergaben, dass das Larvalgewicht von *E. acraea* positiv mit der β-Glucosidase-Aktivität gegenüber Aucubin korrelierte, während das Larvalgewicht von *J. coenia* nicht mit der β-Glucosidase-Aktivität (für keines der beiden Substrate) korreliert war. Beide Arten unterschieden sich zwar hinsichtlich der 4-Nitrophenyl-β-D-Glucose hydrolysierenden β-Glucosidase-Aktivität und auch bezüglich der Quantität der Aucubin-Hydrolyse auf den beiden Wirtspflanzen, aber es scheint, als wären beide Arten gut an Iridoidglycoside angepasst. Vermutlich gibt es bei beiden Arten einen weiteren Mechanismus, der die Toleranz von Iridoidglycosiden ermöglicht. Insbesondere vor dem Hintergrund der hohen β-Glucosidase-Aktivität gegenüber Aucubin, die sowohl bei dem Generalisten als auch bei dem Spezialisten zu finden sind, erscheint dies plausibel.

5.4 Generalisten und Iridoidglycosidspezialist im Vergleich

Beim Vergleich der β-Glucosidase aller untersuchten Generalisten mit der des Iridoidglycosidspezialisten zeichnete sich folgendes Bild ab: Alle von mir untersuchten Lepidopterenarten wiesen zwei β-Glucosidase-Isoformen auf, die 4-Nitrophenyl-β-D-Glucose als Substrat umsetzen. Die Michaelis-Konstante für 4-Nitrophenyl-β-D-Glucose der hochaffinen β-Glucosidasen lag bei allen vier Arten zwischen 0,2 mM und 1,0 mM, wobei Michaelis-Konstanten zwischen 0,4 mM und 0,5 mM am häufigsten vorkamen. Die Variabilität könnte durch verschiedene Allele zustande kommen. Da die β-Glucosidasen nicht

weiter aufgereinigt wurden, könnten dadurch ebenfalls Ungenauigkeiten bei der Bestimmung der Michaelis-Menten Konstanten aufgetreten sein. Die ermittelten Michaelis-Konstanten für 4-Nitrophenyl-β-D-Glucose sind hierbei gut mit den Konstanten anderer Lepidopteren vergleichbar, deren β-Glucosidasen aufgereinigt und biochemisch charakterisiert wurden (Azevedo et al. 2003; Marana et al. 2000; Pratviel-Sosa et al. 1987; Santos and Terra 1985).

Des Weiteren bestimmte ich die Substrataffinität von β-Glucosidase gegenüber Aucubin. Erstaunlicherweise lagen die Michaelis-Konstanten für die β-Glucosidasen der Generalisten *G. incorrupta* und *E. acraea* auch in einer ähnlichen Größenordnung wie die des Iridoidglycosidspezialisten *J. coenia*. Sie schwankten zwischen 1,1 mM und 1,9 mM, wobei die Konstanten von *E. acraea* eher niedrigere und die von *J. coenia* eher höhere Werte aufwiesen. Nur eine Michaelis-Konstante der β-Glucosidase von *G. incorrupta* für Aucubin lag mit 3,9 mM deutlich höher als die anderen Konstanten. Soweit mir bekannt ist, handelt es sich hier um eine der ersten Arbeit, in der die Substrataffinität von β-Glucosidasen zu Iridoidglycosiden untersucht worden ist. Aus diesem Grund liegen bislang noch keine Vergleichswerte aus anderen Studien vor. Hinsichtlich der Substrataffinität von β-Glucosidase zu anderen toxischen Glycosiden haben verschiedene Autoren darauf hingewiesen, dass bei Spezialisten (durch Selektionsdruck) eine β-Glucosidase mit einer reduzierten Affinität zu toxischen Glycosiden evolvieren könnte, die Fitnessvorteile durch die Verringerung der Autointoxikation bietet (Ferreira et al., 1997, Marana et al., 2000). In Bezug auf die Anpassung an Iridoidglycoside scheint dies für das Enzym β-Glucosidase des Iridoidglycosidspezialisten nicht der Fall zu sein. Die ähnlichen Michaelis-Konstanten zeigen vielmehr, dass bei allen drei Arten mit der Aucubin hydrolysierenden β-Glucosidase die Iridoide mit einer ähnlichen Affinität umgesetzt werden.

Welchen Einfluss haben nun die einzelnen Wirtspflanzen auf die β-Glucosidase-Aktivität der Generalisten und des Spezialisten? Allen drei Generalisten ist gemeinsam, dass die mit dem Substrat 4-Nitrophenyl-β-D-Glucose bestimmte β-Glucosidase-Aktivität mit steigenden Iridoidglycosidkonzentrationen in der Pflanze geringer wurde. Offensichtlich handelt es sich bei den drei Arctiiden-Arten um eine adaptive Regulation der β-Glucosidase-Aktivität, die mit steigenden Iridoidglycosidkonzentrationen in den Wirtspflanzen einherging. Hierbei spielte es keine Rolle, ob die Larven nur für zwei Wochen (*S. virginica*) oder während ihrer gesamten Larvalzeit (*E. acraea* und *G. incorrupta*) auf den Pflanzen gehalten wurden. Für *G. incorrupta* konnte ich zeigen, dass die Fähigkeit, die mit 4-Nitrophenyl-β-D-Glucose

bestimmte β-Glucosidase-Aktivität zu reduzieren, mit einem höheren Larvalgewicht einhergeht. Das deutet daraufhin, dass die Reduzierung von β-Glucosidase-Aktivität den Raupen Fitnessvorteile bringt. Eine adaptive Regulation der β-Glucosidase-Aktivität als Reaktion auf hohe Glycosidkonzentrationen in der Nahrung ist als Anpassung auch schon für andere Insektenarten gezeigt worden (Desroches et al., 1997, Ferreira et al., 1997, Lindroth, 1988, Mainguet et al., 2000). Im Gegensatz dazu behielt der Iridoidglycosidspezialist *J. coenia* seine mit 4-Nitrophenyl-β-D-Glucose ermittelte β-Glucosidase-Aktivität nicht nur bei, sondern steigerte sie noch, wenn er auf der Wirtspflanzenart mit hohen Iridoidglycosidkonzentrationen, *P. lanceolata*, frass.

Hinsichtlich der Fähigkeit Aucubin zu hydrolysieren, machte ich eine interessante Beobachtung. Tatsächlich fand ich eine gut messbare β-Glucosidase-Aktivität gegenüber Aucubin nur dann, wenn die Raupen (*J. coenia, E. acraea* und *G. incorrupta*) während ihrer gesamten Larvalzeit auf der Wirtspflanzenart mit hohen Iridoidglycosidkonzentrationen, nämlich *P. lanceolata*, gehalten wurden. Bei den Raupen von *S. virginica*, die nur zwei Wochen auf *P. lanceolata* gehalten wurden, konnte ich keine signifikanten Unterschiede zwischen den Wirtspflanzen hinsichtlich der β-Glucosidase-Aktivität gegenüber Aucubin finden. Im Vergleich dazu war die β-Glucosidase-Aktivität bei dem Spezialisten *J. coenia* und auch bei den Generalisten *E. acraea* und *G. incorrupta* signifikant erhöht, wenn die Raupen auf *P. lanceolata* gehalten wurden.

Für *J. coenia* wurde schon früher angenommen, dass ein großer Teil der mit der Nahrung aufgenommenen Iridoidglycoside metabolisiert statt sequestriert wird (Bowers & Collinge, 1992). Dies lässt sich durch die von mir gemessene hohe β-Glucosidase-Aktivität gegenüber Aucubin belegen. Die hohe β-Glucosidase-Aktivität gegenüber Aucubin könnte erklären, weshalb Bowers & Collinge (1992) keine intakten Iridoidglycoside im Kot von *J. coenia* nachweisen konnten. Das ist insofern erstaunlich, weil die Hydrolyse von Aucubin eine Denaturierung von Protein und damit eine Verschlechterung der Proteinmenge und -qualität zur Folge hat (Kim et al., 2000, Konno et al., 1999). Protein ist aber essentiell für das Larvalwachstum und zu geringe Proteinmengen aufgrund einer geringeren Verfügbarkeit führen im Allgemeinen zu einer verlängerten Larvalentwicklung und einer reduzierten Fitness (Felton et al., 1992). Während für den Spezialisten *J. coenia* keine geringere Performance auf *P. lanceolata* beobachtet wurde, wogen die Raupen des Nahrungsgeneralisten *G. incorrupta* auf *P. lanceolata* deutlich weniger und ihre Larvalentwicklung war signifikant länger als bei

den Raupen, die auf dem iridoidglycosidfreien *T. officinale* gehalten wurden. Diese schlechtere Performance von *G. incorrupta* Raupen lässt sich auf die β-Glucosidase-Aktivität gegenüber Aucubin zurückführen, die signifikant negativ mit dem Larvalgewicht korreliert war. Somit zeigt sich zumindest für die Raupen von *G. incorrupta*, dass die β-Glucosidase-Aktivität gegenüber Aucubin offensichtlich die Fitness der Larven negativ beeinflusst.

Im Vergleich zu *G. incorrupta* war bei den Raupen von *E. acraea* die β-Glucosidase-Aktivität gegenüber Aucubin positiv mit dem Larvalgewicht korreliert. Eine mögliche Erklärung hierfür wäre, dass beide Arten unterschiedlich auf dauerhaft hohe Konzentrationen von Iridoidglycosiden in der Nahrung reagieren. Obwohl von beiden Bärenspinner-Arten bekannt ist, dass Iridoidglycoside stimulierend auf die Nahrungsaufnahme wirken, könnte es sein, dass die *G. incorrupta* und *E. acraea* rein physiologisch unterschiedlich gut an Iridoidglycoside angepasst sind. Allgemein sind beide Arten sehr polyphag und wechseln ihre Wirtspflanzen häufig, wobei sie auch Pflanzen mit geringerem Nährwert in ihrem Wirtspflanzenspektrum haben (Singer & Bernays, 2008, Singer et al., 2004a, Singer et al., 2004b, Singer & Stireman, 2001). Neben einem Wechsel der Wirtspflanze wäre es zudem möglich, dass *E. acraea* Raupen auf verringerte Proteinmengen in der Nahrung wie *J. coenia* (Camara, 1997) mit einer kompensatorischen Nahrungsaufnahme reagieren und größere Mengen an Pflanzenmaterial aufnehmen, während dies bei *G. incorrupta* möglicherweise nicht der Fall ist. Hier sind weitere Experimente nötig, um diesen Sachverhalt zu klären. Bemerkenswert ist auch, dass bei beiden Generalisten eine Aucubin hydrolisierende β-Glucosidase auf *P. lanceolata* induziert wurde, während dies bei den Raupen von *S. virginica* auf *P. lanceolata* nicht der Fall war. Möglicherweise spielt hier die Dauer eine Rolle, das heißt, wie lange die Tiere ausschließlich auf einer Wirtspflanze gehalten wurden. Die Induktion einer Aucubin hydrolysierenden β-Glucosidase widerspricht zudem der beobachteten Reduzierung der 4-Nitrophenyl-β-D-Glucose-hydrolysierender β-Glucosidase, die ich ja bei allen drei Generalisten auf *P. lanceolata* gefunden habe.

Möglicherweise hängt die Aucubin hydrolysierenden β-Glucosidase auf eine bestimmte Art und Weise mit der Pflanze *P. lanceolata* zusammen. Es wäre potentiell möglich, dass es sich bei allen drei Arten *J. coenia, E. acraea* und *G. incorrupta* nicht um ihre eigene β-Glucosidasen (tierischen Ursprungs) handelt, sondern um ein Enzym, was von der Darmflora produziert wird und nur durch hohe Iridoidglycosidmengen während eines

längeren Zeitraums induziert wird. Es ist bekannt, dass die Darmflora bei Insekten einen Einfluss auf die Anpassung an Pflanzeninhaltsstoffe hat (Dowd, 1992). Es konnte gezeigt werden, dass die Darmorganismen außerdem zu einem gewissen Grad von den Wirtspflanzen abhängen, auf denen die herbivoren Insekten leben (Broderick et al., 2004, Dillon & Dillon, 2004, Spiteller et al., 2000). Handelt es sich bei der Aucubin hydrolysierenden β-Glucosidase tatsächlich um ein Enzym aus der Darmflora, die wiederum von der Wirtspflanze abhängig ist, so könnte dies auch erklären, weshalb die Michaeliskonstanten aller drei Arten so ähnlich sind. Die hohe Aktivität gegenüber Aucubin bei dem Iridoidglycosidspezialisten *J. coenia* wie auch bei dem Generalisten *E. acraea* lassen außerdem darauf schließen, dass beide Arten über einen oder mehrere weitere Anpassungsmechanismen verfügen müssen, um Iridoidglycoside in der Nahrung zu tolerieren. Die Larven des Iridoidglycosidspezialisten *J. coenia* sequestrieren zudem bis zu 20% ihres Trockengewichts an Iridoidglycosiden (Bowers & Collinge, 1992, Strohmeyer et al., 1998), während *E. acraea* und *G. incorrupta* deutlich geringere Iridoidglycosidmengen sequestrieren (Bowers, persönliche Mitteilung). Aufgrund der hohen Kapazität von *J. coenia*, Iridoidglycoside zu sequestrieren, kommen möglicherweise geringere Iridoidglycosidkonzentrationen im Darm mit β-Glucosidase in Kontakt. Das heißt, es werden auch geringere Mengen durch eine geringere Maximalaktivität des Enzyms hydrolysiert. Des Weiteren wäre es möglich, dass sich ähnlich wie bei anderen Lepidopterenarten auf einer iridoidglycosidhaltigen Wirtspflanze ein einfacher Mechanismus entwickelt hat, der durch das Abpuffern der Aglykone die Proteindenaturierung in der Nahrung verhindert. Verschiedene Studien (Konno et al., 1996; Konno et al., 1997) haben gezeigt, dass diverse Arten aus mehreren Schmetterlingsfamilien als Anpassung an Iridoide Glycin in den Darm sezernieren, das mit den durch Hydrolyse freigesetzten, proteindenaturierenden Aglykonen interagiert und damit als einfaches Schutzmolekül fungiert.

5.5 Ist β-Glucosidase ein Schlüsselenzym für die Anpassung von Insekten an pflanzliche Iridoidglycoside?

Die Ergebnisse meiner Arbeit weisen darauf hin, dass β-Glucosidase bei dem untersuchten Iridoidglycosidspezialisten *J. coenia* nicht das Schlüsselenzym ist, welches die Anpassung an Iridoidglycoside ermöglicht. Für keines der beiden Substrate war die β-Glucosidase-Aktivität bei *J. coenia* mit dem Larvalgewicht korreliert, was daraufhin deutet, dass β-Glucosidase eher einen geringen Einfluss auf die Larvalentwicklung bzw. auf die Fitness von *J. coenia* hat.

Andererseits könnte β-Glucosidase bei höheren Iridoidglycosidkonzentrationen in der Nahrung durchaus einen Einfluss auf die Fitness haben. In zwei Studien wurde gezeigt, dass hohe Iridoidglycosidkonzentrationen negative Effekte auf die Larven haben (Adler et al., 1995, Camara, 1997). Bei hohen Iridoidglycosidkonzentrationen (um die 10%) in der Nahrung wird die Effizienz reduziert, mit der Nährstoffe aus der konsumierten Futtermenge extrahiert und resorbiert werden, wodurch die Gewichtszunahme pro konsumierter Futtermenge sinkt (Camara, 1997). Die Raupen begegnen dem aber kompensatorisch durch eine erhöhte Futteraufnahme. Bei hohen Aucubinkonzentrationen in *P. lanceolata*-Genotypen überleben hingegen signifikant weniger Raupen als auf Genotypen mit geringeren Aucubinmengen (Adler et al., 1995). Eine hohe β-Glucosidase-Aktivität bei hohen Aucubinkonzentrationen könnte also der entscheidende Faktor sein, der bei Raupen die Mortalität stark erhöht.

Im Gegensatz zu *J. coenia* reduzieren alle drei Generalisten die mit 4-Nitrophenyl-β-D-Glucose bestimmte β-Glucosidase-Aktivität, wenn sie *P. lanceolata* konsumierten. Die Fähigkeit, die β-Glucosidase-Aktivität zu regulieren, spielt zumindest bei *G. incorrupta* eine bedeutende Rolle und bewirkt eine höhere die Gewichtszunahme bei den Raupen. Die Hydrolyse von Aucubin durch β-Glucosidase führt bei *G. incorrupta* Raupen hingegen zu einem niedrigeren Larvalgewicht. Vermutlich spielt β-Glucosidase also bei Generalisten eine wichtige Rolle für die Toleranz von pflanzlichen Glycosiden in der Nahrung. Die adaptive Regulation der β-Glucosidase-Aktivität bei allen drei polyphagen Arten deutet daraufhin, dass es sich hier um einen weit verbreiteten Mechanismus handelt, der vermutlich insbesondere eine kurzzeitige Nutzung der Wirtspflanze ohne schädliche Effekte gewährleistet. Andererseits weisen die Ergebnisse für *E. acraea* und *J. coenia* darauf hin, dass neben β-Glucosidase ein weiterer oder mehrere weitere Mechanismen zur Verfügung stehen müssen, welche die hohe β-Glucosidase-Aktivität gegenüber Aucubin ausbalancieren. Dieser potentielle Entgiftungsmechanismus scheint – wenigstens für *J. coenia* - einen „threshold“ bei hohen Iridoidglycosidkonzentrationen zu besitzen (Adler et al., 1995). Unter diesen Umständen hat die hohe β-Glucosidase-Aktivität gegenüber Aucubin auch für den Iridoidglycosidspezialisten *J. coenia* Fitnesskonsequenzen.

Um die Frage von Coevolution zwischen den von mir untersuchten Arten und iridoidglycosidhaltigen Pflanzen aufzugreifen, so deuten die hohe Variabilität der Michaelis-Konstanten auf biochemischer Ebene daraufhin, dass eine höhere phenotypische Plastizität

bei allen Arten vermutlich die Toleranz von hohen Iridoidglycosidkonzentrationen innerhalb einer Population verbessert. Dies gilt ebenfalls für die Fähigkeit, β-Glucosidase in Reaktion auf hohe Glycosidkonzentrationen in der Pflanze zu reduzieren. Andererseits bringt die unklare Herkunft der Aucubin hydrolysierende β-Glucosidase Schwierigkeiten mit sich, wenn man sie im Sinne von Coevolution interpretieren möchte. Es bleibt weiterhin eine interessante und offene Frage, in wie weit verschiedene trophische Ebenen bei der Anpassung an Iridoidglycoside eine Rolle spielen. Neben den Prädatoren, herbivoren Konkurrenten und Parasitoiden kommt nun möglicherweise auch noch die Ebene der Darmflora hinzu, die in Abhängigkeit von der Wirtspflanze die Anpassung an deren Inhaltsstoffe mit beeinflusst.

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Danksagung

An erster Stelle möchte ich der Betreuerin meiner Dissertation, Frau Prof. Susanne Dobler danken. Sie hat mich an das interessante Thema „Chemische Ökologie“ herangeführt und mir damit ein spannendes Feld für eigene Fragestellungen und Untersuchungen aufgezeigt. Insbesondere danke ich ihr für viele interessante Diskussionen, herzliches Lachen und konstruktive Kritik, die mich in der Entwicklung eines eigenen Arbeitsstils maßgeblich weiter gebracht haben.

Des Weiteren danke ich Frau Prof. M. Deane Bowers, die mich während meines Auslandaufenthaltes in Boulder im Jahr 2007 sehr herzlich in ihre Arbeitsgruppe mit aufgenommen hat, wo sie mir grundlegende Kenntnisse in der Iridoidglycosidanalytik an der GC vermittelte. Sie hat mich über die letzten Jahre fortwährend mit Insektennachschub versorgt, meine englischen Manuskripte korrigiert und diese nachhaltig durch ihre konstruktive Kritik verbessert.

Ebenfalls möchte ich Herrn Prof. Reinhard Lieberei danken, der mich schon während meines Studiums für das Thema „Biochemische Ökologie der Pflanzen“ begeistert hat. Weiterhin hat er mir bei Fragen sachkundigst weitergeholfen und mich in verschiedensten Situationen mit Rat und Tat unterstützt.

Weiteren Menschen, ohne die ich die Arbeit sicher nicht hätte fertig stellen können, danke ich in unsortierter Reihenfolge und hoffe, dass die Liste weitgehend vollständig ist:

Georg Petschenka für Freundschaft, unermüdliche Diskussionen und wissenschaftlichen Austausch über so ziemlich alles, was man mal in nächster Zeit unbedingt in Angriff nehmen sollte und natürlich für seine unendlichen Kenntnisse bei der Raupenpflege und Falterzucht,

Dr. Gregor Kölsch für Hilfestellungen und gute Diskussionen zu allem, was mich so zwischen Labortür und seinem Raum so bewegt hat,

Herr Prof. Lothar Renwrantz für sein allzeit offenes und interessiertes Ohr zu verschiedensten Aspekten meiner Arbeit,

Dr. Gerhard Dörfler, der mich sowohl während meines Studiums als auch während meiner Diplomarbeit und Dissertation immer wieder aufgemöbelt hat, wenn ich mal einen neuen Schub an Motivation gebraucht habe,

Dr. Helmut Kassner , der mich während meiner Dissertation mit seiner Sachkenntnis und viel Arbeitszeit bei der Aufreinigung von Iridoidglycosiden unterstützt hat,

der Insektenbörse ACTIAS, durch die ich Nachschub von verschiedenen interessanten Schmetterlingsarten erhalten habe,

Dr. Ellen Schulz für Freundschaft, Frauenpower und viele wissenschaftliche Gespräche.

Ein herzliches Dankeschön geht auch an alle anderen Freunde und Kollegen in der Arbeitsgruppe Dobler, die mich während meiner Dissertation begleitet haben. Ich danke Irina Flat für ihre unermüdliche Hilfe und Zuarbeit in der letzten Zeit meiner Dissertation, Karin Meyer für langjährige Unterstützung in jeder Hinsicht, Hatice Capar für eine gute Zimmer-Nachbarschaft, Saskia Lieberei für eine schöne gemeinsame Studien- und Doktorandenzeit, Vera Wagschal für alle Hinweise zum guten und richtigen Arbeiten im Labor (und nicht zu vergessen für eimerweise Löwenzahn und Wegerich), Christiane Bramer für die immer wiederkehrenden leckeren Gebäckstücke und entspannte Unterhaltung, Christian Baden, Regina Jäckel, Julia Offe, Jens Krobbach, Ingo Naberhaus, Peter Iglauer, Birgit und Jan-Jakob für gute Zusammenarbeit,

und natürlich danke ich meiner Mittagsessensgruppe, mit denen ich über Jahre ein solides tägliches Kickertraining in der Ponybar genossen und viele weitere schöne Stunden verbracht habe: Jan, Ellen, Tina und Fredde, Volker, Juan-Pablo, Ivan, Georg, Simone, Anja und Thomas, diversen Ethos und manchen Ganzhörner und allen anderen, die immer mal wieder mitgekommen sind.

Außerdem danke ich meinen langjährigen Freunden Konni Petersen und Dirk Wagner für Aufmunterungen, Zuverlässigkeit in jeder Lebenslage und viele schöne Zeiten in den letzten zehn oder elf Jahren, und Jens Oldeland für ein tolles gemeinsames Biostudium,

ich danke meiner Familie für Unterstützung und Zuspruch jeglicher Art während meines gesamten Studiums und meiner Promotionszeit,

und ganz besonders danke ich Jan, der für mich – außer dass er mein bester Freund ist – auch der Mann und Biologe an meiner Seite ist, von dem ich mir immer gewünscht habe, dass es ihn gibt.

Und nicht zuletzt möchte ich dem DAAD für die Bewilligung eines Stipendiums für einen Forschungsaufenthalt in den USA danken.