ALPHA-AMYLASE, CORTISOL, AND PUPILLARY RESPONSES TO SOCIAL AND NON-SOCIAL DYNAMIC SCENES IN YOUNG CHILDREN WITH AUTISM SPECTRUM DISORDER

BY

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Abstract

Social dysfunction is a hallmark feature of Autism Spectrum Disorder (ASD). However, since the initial description of ASD by Kanner (1943) it has been recognized that the disorder may manifest from a more basic neuropsychological deficit in attention and/or arousal, and previous studies have found altered autonomic and attentional responses during both baseline conditions and in response to socially-relevant stimuli in those with ASD. Based on this line of inquiry, we recently used eye-tracking technology to examine visual scanning and pupillary responses and found a larger tonic (baseline) pupil size (Anderson & Colombo, 2009) and altered phasic (task-specific) pupillary responses to human faces, with no group-based differences in visual scanning (Anderson, Colombo, & Shaddy, 2006) in 2-5 year old children with ASD compared to age-matched controls. To replicate and extend these previous results, children (20 – 72 months of age) with ASD (n = 12), along with Down syndrome (DS; n = 9), and typically-developing (TD; n = 11) age-matched controls were presented with a social and a non-social dynamic and multimodal video clip. Each stimulus was presented for 10 minutes on two separate testing days; location of gaze and pupil size was recorded, along with salivary measures of alpha-amylase and cortisol. Tonic measures of pupil size, AA and cortisol were also recorded during a baseline period. The ASD group was significantly distinguished in group-based analyses from both the DS and TD groups through (a) a larger tonic pupil size, (b) lower tonic levels of AA, which were significantly related to tonic pupil size, and (c) increased phasic pupil responses to the social stimulus. These findings provide replication of our previous investigations and a unique finding of lower AA levels in the ASD group. These results may provide clues about underlying norepinephrine system pathology in ASD, and the potential of
non-invasive measures of pupil size and salivary AA in the early identification and screening of the disorder.
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Social dysfunction is a hallmark feature of Autism Spectrum Disorder (ASD) and is one of the three core behavioral deficits used to diagnose the disorder; the core dysfunctions include deficits in social interaction, communication, and the presence of repetitive or stereotyped behaviors (American Psychiatric Association [APA], 1994). In addition, there are a number of other fundamental impairments and deficits demonstrated in persons with ASD. Persons with ASD have shown deficits in social orienting, human face processing, emotion processing, executive function, theory of mind, abstraction, central coherence, joint attention, attentional orienting, sustained attention, sensory modulation, arousal, and motor disturbances (see Bailey, Phillips, & Rutter, 1996; Joseph, 1999; Klinger, Dawson, & Renner, 2003, for reviews). Neurological investigations have generated both neuroanatomical and neurochemical findings in persons with ASD. The neuroanatomical findings suggest impairments in the brainstem, cerebellum, ventricles, amygdala, hippocampus, limbic cortex, temporal lobe, parietal lobe, frontal lobe, and corpus callosum to be involved in ASD (see Acosta & Pearl, 2004; Bauman & Kemper, 1985; Brambilla, Hardan, di Nemi, Perez, Soares, & Barale, 2003; Palmen, van Engeland, Hof, & Schmitz, 2004, for reviews). Neurochemical investigations have found alterations in epinephrine, norepinephrine (NE), serotonin (5-HT), acetycholine (ACh), glutamate (Glu), and gamma-aminobutryric acid (GABA) systems in those with ASD (see, Cook, 1990; Volkmar & Anderson, 1989, for reviews).

Neuropathologic Models of Autism Spectrum Disorder

Since the initial description of ASD by Kanner (1943), it has been recognized that the symptoms of the disorder may manifest from a more basic neuropsychological deficit in...
attention and/or arousal (e.g., Ornitz, 1969; Dawson & Lewy, 1989). Since this time, investigations at multiple-levels of analysis have led to a heterogeneous range of behavioral, cognitive, and neurological findings in ASD. However, despite the fact that ASD is generally accepted as a neurodevelopmental disorder, the neuropathologic origin of this disorder remains elusive and a wide array of theories on the neurological origin of ASD have been posited. Contemporary neuropathologic theories, based on results from post-mortem and neuroimaging studies, have focused on the limbic system, cerebellum, and brainstem as being the source of ASD neuropathology, with attention and arousal continuing to emerge as a consistent theme in these contemporary theories.

**Limbic System**

**Limbic system.** One of the most consistent neuroanatomical findings from postmortem examinations in ASD is a reduced cell size and increased cell density within the hippocampus, amygdala, mammillary bodies, anterior cingulate gyrus, and septum, all of which make up a major portion of the forebrain limbic system (Bauman & Kemper, 1984, 1985, 1987, 1990), and a decreased number of neurons within the amygdala (Schumann & Amaral, 2006). Bauman and Kemper (1998) suggested that the nature of the limbic system impairment in ASD is consistent with a curtailed development of these structures, as small, densely packed neurons in the limbic system are typically seen during earlier stages of development before they have reached adult proportions. In addition, early acquired lesions to newborn rhesus monkeys within the amygdala, hippocampus, and adjacent cortical areas have been found to produce similar motor stereotypies, memory (e.g., impaired explicit memory, which is rapid one-trial learning, and preserved habitual memory, which is acquired by repeated presentation of the same object), and socioemotional (e.g., lack of social initiation, increased irritability in social situations, decreased
social interaction, lack of eye contact) deficits as those found in persons with ASD (Bachevalier, 1994). Therefore, it has been suggested that prenatal impairment within the limbic system causes a cascade of developmental effects that may include further neural impairment of cortical structures via corticolimbic connections (Bauman & Kemper, 1998), and additional neural compensatory effects that may include a progressive increase in brain weight that could reflect increased neurogenesis, decreased apoptosis, and decreased synaptic pruning (Bauman & Kemper, 2005). Bauman and Kemper (1998) suggested that prenatal impairment to the forebrain limbic structures could disrupt the acquisition and interpretation of information resulting in the cognitive, language, and social interaction deficits found in those with ASD. In addition they suggested that the preservation of habit memory would lead to a need for sameness and preoccupied interests, and impaired explicit memory would lead to an inability to acquire social, language, and cognitive skills.

**Amygdala.** A more recent limbic system theory of ASD has focused specifically on the amygdala (Baron-Cohen et al., 2000) and is based on the role that the amygdala is believed to play in social cognition (Brothers, 1990). Experimental lesions of the amygdala have been found to result in social avoidance, lack of eye contact, absence of facial expressions and emotional reactions, abnormal movement patterns and stereotypies, increased aggression, loss of fear, and examination of objects by mouth and smell in non-human primates, with the greatest social effects occurring when lesions were sustained during the neonatal period (see Baron-Cohen et al., 2000; Sweeten, Posey, Shekhar, & McDougle, 2002, for reviews). Studies of human amygdala lesions and functional magnetic resonance imaging (fMRI) also support a role for the amygdala in social behavior. Persons with amygdala lesions have been shown to display social deficits similar to those found in ASD (see Sweeten et al., 2002, for a review), and increased
amygdala activation has been found to be associated with the presentation of facial expressions and interpretation of gaze direction (see Pelphrey, Adolphs, & Morris, 2004, for a review). Neuroimaging examinations have further implicated the presence of amygdala impairments in persons with ASD, although the results of these investigations have been inconsistent. For example, some MRI examinations have found reduced (Aylward et al., 1999; Nacewicz et al., 2006) and increased (Abell et al., 1999; Howard et al., 2000; Sparks et al., 2002) amygdala volumes, while others have found no differences (Haznedar et al., 2000; Saitoh, Courchesne, Egaas, Lincoln, & Schreibman, 1995). Although age has been found to have a significant effect on amygdala volume in ASD, with studies of children with ASD showing enlarged amygdala volume, adult ASD studies have found no differences (Stanfield et al., 2008). Thus age differences may explain the volumetric discrepancies in amygdala imaging studies in ASD. In addition, fMRI examinations in ASD have revealed increased amygdala activation to human faces (Kleinhans et al., 2009) and while fixating within the eye region of human faces (Dalton et al., 2005), while other studies have found less amygdala activation to human faces depicting fear (Ashwin, Baron-Cohen, Wheelwright, O’Riordan, & Bullmore, 2007), when inferring mental states from the eye region of human faces (Baron-Cohen et al., 1999) and when inferring gender (Critchley et al., 2000) in persons with ASD.

A more recent line of inquiry, has suggested that the amygdala may not be the source of ASD social deficits (Amaral & Corbett, 2003; Bauman, Lavenex, Mason, Capitanio, & Amaral, 2004; Dziobek, Fleck, Rogers, Wolf, & Convit, 2006). Instead, based on non-human primate studies of bilateral lesions to the amygdala in adults and neonates, it has been suggested that the role of the amygdala may be to evaluate the environment for possible threats, as impaired primates have been shown to display decreased fear responses to fear-provoking objects and
increased fear responses during novel social interactions, with preserved social behaviors (Amaral & Corbett, 2003; Bauman et al., 2004; Prather et al., 2001). Thus, while the role of the amygdala in ASD is unclear, these recent investigations suggest that impairment within this structure may be related to abnormal fears and anxiety rather than social deficits. However, further investigations are needed to determine the role of the amygdala in ASD and to determine if amygdala impairment might be a core dysfunction or may be a secondary consequence of other neural impairments.

**Cerebellum and Brainstem**

Since the initial description of ASD by Kanner (1943), it has been recognized that the symptoms of ASD may manifest from a more basic neuropsychological deficit in arousal and attention (e.g., Courchesne et al., 1995; Dawson & Lewy, 1989; Ornitz, 1969). This is because appropriate levels of arousal and the allocation of attention are necessary precursors to higher cognitive functions (e.g., Colombo, 2001; James, 1890; Lane & Pearson, 1982); therefore, attentional and/or arousal deficits could interfere with an individual’s with ASD ability to attend, process, and interact with the environment and result in a failure to acquire normative skills (e.g., Bryson, Landry, & Wainwright, 1997; Dawson & Lewy, 1989). In addition, the neural systems that underlie arousal and attention (e.g., the cerebellum, pons and medulla), can alter neuronal activity in areas responsible for sensory, motor, and emotional processing (Posner & Raichle, 1994). Consequently, structural examinations have revealed consistent abnormalities within the cerebellum and brainstem in persons with ASD, and these observations have led to several theories on how cerebellum and brainstem impairment may be involved in generating the behavioral, cognitive, and neural effects found in ASD.
Cerebellum. The majority of postmortem examinations investigating the cerebellum in ASD have revealed a significant reduction in the number of Purkinje cells (Bailey et al., 1998; Bauman, Filipek, & Kemper, 1997; Bauman & Kemper, 1985, 1990, 1994, 1998; Fatemi et al., 2002; Ritvo et al., 1986; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2004; Weidenheim et al., 2001; Williams, Hauser, Purpura, DeLong, & Swisher, 1980), however a recent study using an updated stereology method found only half of the ASD brains (three out of six) to contain this reduction (Whitney, Kemper, Bauman, Rosene, & Blatt, 2008). MRI examinations have also revealed cerebellum impairment in those with ASD, although the direction and results of these investigations have been varied. Some studies have revealed a decreased size of the cerebellar vermis lobules VI-VIII (Courchesne et al., 2001; Courchesne, Yeung-Courchesne, Press, Hesselink, & Jernigan, 1988; Kaufmann et al., 2003; Muurakami, Courchesne, Press, Yeung-Courchesne, & Hesselink, 1989; Piven et al., 1992; Rojas et al., 2006) and lobules VIII – X (Hashimoto, Tayama, Miyazaki, Murakawa, & Kuroda, 1993; Levitt et al., 1999; Rojas et al., 2006), reduced vermis volume (Scott, Schumann, Goodlin-Jones, & Amaral, 2009), increased (Hardan, Minshew, Hareski, & Keshavan, 2001) and reduced cerebellar volume (Hallahan et al., 2009), and proportionately decreased (Gaffne, Tsai, Kuperman, & Minchin, 1987) and increased cerebellum size (Herbert et al., 2003; Sparks et al., 2002) in ASD; other studies have found no differences (Garber & Ritvo, 1992; Hashimoto et al., 1993; Hashimoto, Murakawa, Miyazaki, Tayama, & Kuroda, 1992; Holttum, Minshew, Sanders, & Phillips, 1992; Kleiman, Neff, & Rosman, 1992; Manes, Piven, Vrancic, Nanclares, Plebst, & Starstein, 1999). However, a recent meta-analysis of MRI investigations suggests that the heterogeneity of these results may be due to age; Stanfield et al (2008) found that as the mental-
and chronological-age of the subjects increased the reduction in vermal lobule VI-VII volumes of subjects with ASD became less evident.

Functionally, the cerebellum is believed to play a role in attentional orienting (Akshoomoff & Courchesne, 1992; Allen, Buxton, Wong, & Courchesne, 1997) and further evidence for cerebellum involvement in ASD comes from studies revealing that persons with ASD have slowed orienting responses to target stimuli that occur at intervals of 500 msec or less, but show typical responses to target stimuli that occur after 700 msec (e.g. Casey, Gordon, Mannheim, & Rumsey, 1993; Harris, Courchesne, Townsend, Carper, & Lord, 1999; Townsend et al., 1999; Townsend, Harris, & Courchesne, 1996; Townsend & Courchesne, 1994). In addition, functional neuroimaging examinations reveal reduced cerebellar activation to target stimuli (Allen & Courchesne, 2003), while listening to tones (Muller et al., 1999), and during tasks requiring the judgment of emotional expressions (Critchley et al., 2000).

It has been suggested that the reduction in inhibitory Purkinje cells within the cerebellum could lead to frontal and temporal lobe impairments through excessive excitatory output from the cerebellum during development (Carper & Courchesne, 2000; Courchesne, 2004). This could lead to altered minicolumns within frontal and temporal regions (Casanova, Buxhoeveden, Switala, & Roy, 2002). Minicolumns have been found to be smaller and more narrow in size, but with an increased number in persons with ASD (Buxhoeveden et al., 2006; Casanova et al., 2002, 2006), which likely contributes to the increased frontal lobe volume found in those with the disorder (Buxhoeveden et al., 2006). In addition, larger frontal cortical volume has been shown to be negatively correlated with decreased cerebellar vermis lobules VI and VII volumes in children with ASD, but not in controls (Caper & Courchesne, 2000), implicating a developmental link between these impairments. Thus, cerebellum impairment could affect the
neural pathology in ASD through common neural pathways between the cerebellum and cortical structures (Allen et al., 2005; Middleton & Strick, 2001) and the ability of the cerebellum to influence the development of the cortex (e.g., Quartz & Sejnowski, 1997). Cognitively, cerebellum impairment would lead to slowed orienting early in life impairing joint attention abilities and subsequently other social, language, and cognitive skills (Courchesne, Chisum, & Townsend, 1994).

**Inferior olive.** The inferior olive of the medulla has also been found to be consistently impaired in postmortem examinations of persons with ASD. In particular, olivary neurons have been found to be displaced (Bailey et al., 1998; Bauman & Kemper, 1985, 1994; Rodier, Ingram, Tisdale, Nelson, & Romano, 1996) and atypical in size (Bauman & Kemper, 1985, 1994), and to contain swollen axon terminals (Weidenheim et al., 2001). The inferior olive receives diverse inputs from the thalamus, areas of the brainstem, cerebellum, and spinal cord, but all of the efferent projections are to the cerebellum and are the sole source of climbing fibers, which are fibers that climb the Purkinje cells within the cerebellum (DeZeeuw et al., 1998; Holmes & Stewart, 1908). The nature of the inferior olive impairment in ASD suggests that this impairment most likely occurred prior to cerebellum impairment because pre- or postnatal injury to the cerebellum results in inferior olive cell loss (Greenfield, 1954; Norman, 1940). Therefore, it has been suggested that inferior olive impairment may be the source of ASD neuropathology (Bauman & Kemper, 2005; Welsh, Ahn, & Placantonakis, 2005), causing a reduction in the number of Purkinje cells and subsequent neural and cognitive effects.

**Pons.** Postmortem and neuroimaging examinations have also revealed impairment within the pontine structure in ASD. In postmortem studies, the pons has been found to be atypical in size (Rodier et al., 1996), have an atypical tract within the pontine tegmentum (Bailey...
et al., 1998), and to contain swollen axons terminals (Weidenheim et al., 2001). More specifically, the locus coeruleus (LC) of the pons has been found to be widely dispersed with loosely grouped LC neurons (Bailey et al., 1998) and swollen axon terminals (Weidenheim et al., 2001), although a more recent postmortem study found no LC abnormalities in persons with ASD (Martchek, Thevarkunnel, Bauman, Blatt, & Kemper, 2006). In addition, MRI examinations have revealed the pontine structure to be smaller in persons with ASD than controls (Ciesielski, Harris, Hart, & Pabst, 1997; Craig et al., 2007; Gaffney, Kuperman, Tsai, & Minchin, 1988; Hashimoto et al., 1991, 1995; Hashimoto, Tayama, Miyazaki, Murakawa, & Kuroda, 1993), while other studies have been unable to replicate this result (Elia et al., 2000; Garber & Ritvo, 1992; Hardan et al., 2001; Hashimoto et al., 1992, Hashimoto, Tayama, Miyazaki, Murakawa, Shimakawa et al., 1993; Hsu, Yeung-Courchesne, Courchesne, & Press, 1991; Kleiman et al., 1992; Piven et al., 1992).

The LC of the pons begins development at 3.5 weeks post-conception (Bayer, Altman, Russo, & Zhang, 1993), which is earlier than the amygdala, cerebellum, and inferior olive (see Figure 1). The LC contains the largest group of NE neurons in CNS (Dahlstrom & Fuxe, 1964) and sends projections to almost every region of the brain (e.g., Aston-Jones, Foote, & Bloom, 1984; Foote, Bloom, & Aston-Jones, 1983). In addition, due to the early development of this structure and vast NE innervations, the LC plays a very important role in the regulation of CNS development by providing NE innervations to the CNS throughout embryogenesis (e.g., Coyle, 1977; Schlumpf, Shoemaker, & Bloom, 1980; Sievers, Lolova, Jenner, Klemm, & Sievers, 1981). In fact, LC-NE neurons have been found to penetrate telencephalic structures at approximately seven weeks postconception and play a role in the neurogenesis, migration, and differentiation of cortical structures (Schlumpf et al., 1980). Functionally, the LC is believed to
mediate the processing of information via connections with attentional, memory, sensory, and motor systems (e.g., Aston-Jones & Cohen, 2005; Berridge & Waterhouse, 2003), and is involved in preparing the CNS to be activated by salient stimuli, sleep-wake cycle regulation, nociception, and autonomic responses (e.g., Aston-Jones et al., 1984; Berridge & Waterhouse, 2003). Consequently, sleep-wake cycle deficits (e.g., Honomichl, Goodlin-Jones, Burnham, Gaylor, & Anders, 2002; Richdale & Prior, 1995; Schreck et al., 2004), decreased pain perception (e.g., Baranek & Berkson, 1994; Keintz & Dunn, 1997), and atypical autonomic responses to salient stimuli (e.g., Althaus et al., 2004; Anderson, Colombo, & Shaddy, 2006; Dawson & Lewy, 1989; Hirstein et al., 2001) have been found in those with ASD. Given the structural and functional findings in ASD and the ability of the LC to influence the development of neural structures and cognitive functions found to be impaired in those with the disorder, the LC has been proposed as a possible source of ASD neuropathology (Aston-Jones, Rajkowski, & Cohen, 2000; Dahlstrom, 1989; Mehler & Purpura, 2009).
Figure 1. Estimated timetable of human neurogenesis of structures implicated to be the source of neuropathology in Autism Spectrum Disorders. Adapted by permission from “Timetables of Neurogenesis in the Human Brain Based on Experimentally Determined Patterns in the Rat,” by S. A. Bayer, J. Altman, R. J. Russo, and X. Zhang, 1993, NeuroToxicology, 14. Copyright 1993 by Intox Press, Inc.

Summary and Conclusions

The results of the postmortem, neuroimaging, and cognitive studies presented above implicate the involvement of the limbic system, cerebellum, inferior olive, and pontine structures in producing ASD symptomology. While arguments have been made for each of these structures to be impaired in ASD, the evidence for the primacy of these structures as the source of ASD neuropathology is not compelling; a definitive case has not been established due to (a) inconsistent results, (b) an inability to show how the candidate structure can influence the development of other affected structures and systems, and (c) limited knowledge about typical and atypical neural development of the affected structures. In addition, although emphasis is placed on the structural evidence of the candidate neural regions in ASD, it should be noted, that
evidence for involvement and/or primacy of a particular structure may only be evident in the functional capacity of the structure due to developmental processes and compensatory effects. Therefore, future investigations into each of the structures functions, efferent and afferent connections, and pre- and postnatal development in typical and atypical populations will help to illuminate the role that each of the affected structures play in producing ASD symptomology.

One of the consistent themes that emerge from the current neurological theories presented above is that each of the proposed systems are implicated in producing attentional and/or arousal responses. Therefore, it seems prudent to conduct investigations of the neural systems involved in producing attentional and arousal responses in ASD at multiple levels of analysis; hopefully these will lead to a greater understanding of how the pathology and functional impairments of these systems are involved in producing the disorders’ symptomology. In addition, because lower-order attentional and arousal responses are likely to be present very early in life due to the early development of their underlying structures, these investigations could also lead to the identification of early markers of ASD and further understanding of how these impairments may produce higher-order deficits.

**Study Direction and Document Plan**

It is the goal of my previous and future investigations to use multiple levels of analysis to examine attentional and arousal responses in ASD to gain a better understanding of their presence and involvement in the disorder, their potential as early markers, and the underlying neural systems. Because social dysfunction is a hallmark feature of ASD, one particular line of inquiry is the investigation of attentional and arousal responses to social stimuli. Persons with ASD have unique responses that include decreased time and frequency of looks (e.g., Dawson et al., 2004; Dawson, Meltzoff, Osterling, Rinaldi, & Brown, 1998; Jones, Carr, & Klin, 2008;
Klin, Jones, Schultz, & Cohen, 2002; Maestro et al., 2002; Pelphrey et al., 2002; Riby & Hancock, 2009a; Sweetenham et al., 1998) and atypical autonomic reactions to stimuli with social relevance (e.g., Anderson et al., 2006; Falck-Ytter, 2008; Hirstein, Iversen, & Ramachandran, 2001; Van Hecke et al., 2009). In addition, attentional responses to social stimuli are most commonly reported to differentiate those with ASD during the first year of life (e.g., Adrien et al., 1993; Maestro et al., 2002; Osterling & Dawson, 1994; Werner, Dawson, Osterling, & Dinno, 2000; Zwaigenbaum et al., 2005).

Based on this line of inquiry, we recently used eye-tracking technology to examine the visual scanning and pupillary responses of two to five year old children with ASD, along with mental and chronological age-matched controls, to face (human and animal) and non-face (toy and landscape) static stimuli (Anderson et al., 2006). We were not able to differentiate the groups based on the duration of time they spent looking at the face stimuli. However, we did find the ASD group to have a significant decrease in pupil size from baseline levels to human faces, particularly within the internal features (eyes, nose, and mouth) of the face, while both mental and chronological age-matched controls showed an increase in pupil size and did not differ. In addition, we found the baseline pupil size of the ASD group to be significantly larger than both mental and chronological age-matched controls who did not differ (Anderson & Colombo, 2009). It is the goal of the current study to replicate the results of these previous investigations, while addressing some of the methodological concerns, and extending the findings by investigating neurological systems that may contribute to the atypical pupillary responses during baseline conditions and in response to social stimuli in children with ASD.

The review of literature that follows will focus on the pupillary system and its potential role in ASD. Therefore, a general review of the pupillary system, tonic and phasic influences on
this system, along with the neural structures and neurochemical systems responsible for the control of pupil size will first be provided. This is followed by a review of tonic and phasic pupillary and non-pupillary autonomic responses in ASD and an examination of ASD impairments within neural systems central to the control of pupil size (the NE and hypothalamic systems). Finally, the aim of the current study will be provided along with specific hypotheses and predictions that were made based on this review of literature for the results of the current study.

**Pupillary System**

The pupil is an opening in the iris that allows light to enter the eye and strike the retina. The size of the pupil is determined by an integrated ratio of inhibitory and excitatory activity within the sympathetic and parasympathetic divisions of the autonomic nervous system (ANS). This ratio is determined largely by the characteristics and salience of the visual input. The most common external stimulation that invokes activation of the pupillary system is reflexive pupillary responses to changes in luminance and accommodation efforts. The light reflex is a change in the size of the pupil, which serves to regulate the amount of light entering the eye; thus, the pupil is constricted under conditions of high luminance and dilated under conditions of low luminance (e.g., Andreassi, 2000; Barbur, 2004; Loewenfeld, 1999). The near-reflex or accommodation response is a change in the curvature of the lens, used to control the depth of the visual field; reduction of the pupil size occurs when an object approaches the eye (e.g., Andreassi, 2000; Loewenfeld, 1999).

In addition, there are small changes in pupil size that are not a consequence of the physical properties of the stimulus, but are instead invoked by the psychological content. These small-scale pupillary responses have long been used as an index of the amount of central nervous
system processing that is allocated to a task (e.g., Beatty & Lucero-Wagoner, 2000; Loewenfeld, 1999). However, the magnitude of these task-specific or *phasic* pupillary responses are modulated by the organisms more characteristic or enduring *tonic* state of arousal (e.g., Beatty & Lucero-Wagoner, 2000), and tonic pupil diameter has been used as an indicator of tonic arousal (e.g., Lavie, 1979; Lowenstein & Lowenfeld, 1952; Merritt, Schnyders, Patel, Basner, & O’Neill, 2004; Wilhelm et al., 2001). Finally, it should be noted that pupil size can also be influenced by neurological impairments and pharmacological substances that affect either efferent or afferent pathways (see Beatty & Lucero-Wagoner, 2000; for a review).

**Neuroanatomy of the Pupillary Response**

The size of the pupil is controlled by the tone of two opposing smooth muscles, the sphincter and dilator pupillae. The sphincter pupillae is innervated by the parasympathetic system, mediated by ACh, and causes the iris to constrict. In contrast, the dilator pupillae is innervated by the sympathetic system, mediated by NE, and causes the iris to dilate. A balance between the central sympathetic and parasympathetic divisions of the ANS modulates the diameter of the pupil at any point in time.

**Parasympathetic control of constriction.** The afferent neurons of the parasympathetic system that regulate pupil constriction descend from the retina and send excitatory connections, mediated by Glu, to the pretectal olivary nucleus. The pretectal olivary nucleus then sends excitatory connections, again mediated by Glu, bilaterally to synapse on preganglionic cells within the Edinger-Westphal (EW) nucleus of the midbrain (Gamlin & Clarke, 1995; Hou, Langley, Szabadi, & Bradshaw, 2006; Kourouyan & Horton, 1997). These ACh mediated preganglionic fibers then synapse on the ciliary ganglion (CG). Postganglionic fibers, also
mediated by ACh, then exit the CG and travel via short ciliary fibers to synapse on the sphincter pupillae and cause contraction of the iris (Andreassi, 2000; Beatty & Lucero-Wagoner, 2000; Hou et al., 2006).

**Sympathetic control of dilation.** The afferent neurons of the sympathetic system that regulate pupil dilation descend from the cortex to the posterior hypothalamus (PH) and lateral hypothalamus (LH) (Andreassi, 2000; Beatty & Lucero-Wagoner, 2000; Szabadi & Bradshaw, 1996). The hypothalamus then sends excitatory connections, presumably mediated by histamine and orexin, to the LC, ventrolateral medulla (A1) and ventrolateral pons (A5) (Hou et al., 2006; Stenberg, 2007; Szabadi & Bradshaw, 1996). The LC and A1/A5 nuclei then send NE innervations through the cervico-thoratic spinal cord where they synapse on $\alpha_1$-adrenergic receptors of preganglionic neurons within the ciliospinal center of the Budge (Beatty & Lucero-Wagoner, 2000; Szabadi & Bradshaw, 1996). Preganglionic cholinergic fibers then synapse on cells within the superior cervical ganglion. Postganglionic NE fibers then extend through the carotid plexus and ophthalmic branch of the trigeminal nerve to synapse on $\alpha_1$-adrenergic receptors of the dilator pupillae (Andreassi, 2000; Beatty & Lucero-Wagoner, 2000; Hou et al., 2006).

**Inhibitory influences.** In addition to the central excitatory pathways of the sympathetic and parasympathetic divisions, two central inhibitory pathways also serve to regulate pupil size by providing inhibitory influences on the EW nucleus. First, the LC provides a direct tonic inhibitory influence by releasing NE onto $\alpha_2$-adrenergic receptors within the EW nucleus (Breen, Burde, & Loewy, 1983; Hou et al., 2006; Koss, Gherezghiher, & Nomura, 1984; Szabadi & Bradshaw, 1996). Second, there is a tonic non-noradrenergic inhibitory influence on the EW nucleus, possibly mediated through the release of GABA, from the hypothalamus (Koss et al.,
1984; Koss & Wang, 1972; Li & van den Pol, 2005; Szabadi & Bradshaw, 1996; Wilhelm et al., 2001). Two indirect adrenergic connections serve to enhance the tonic inhibitory influence of the hypothalamus on the EW nucleus. The LC provides an indirect influence on the EW nucleus through activation of $\alpha_1$-adrenergic receptors within the frontal cortex, causing excitatory activation of the hypothalamus to enhance the inhibitory effect on the EW nucleus (Hou et al., 2006; Koss et al., 1984; Lowenstein & Loewenfeld, 1961; Szabadi & Bradshaw, 1996). The A1/A5 areas also influence inhibition of the EW nucleus through activation of $\alpha_1$-adrenergic receptors within the hypothalamus (Guyenet, 1991; Koss et al., 1984; Szabadi & Bradshaw, 1996). Thus, atypical tonic or phasic pupillary responses may reflect a disequilibrium between the parasympathetic and sympathetic divisions of the pupillary system. Figure 2 depicts both the excitatory and inhibitory process involved in the regulation of pupil size.
Figure 2. Excitatory and inhibitory processes involved in the regulation of pupil size. PH = posterior hypothalamus; LH = lateral hypothalamus; OPN = olivary pretectal nucleus; EW = Edinger-Westphal nucleus; LC = locus coeruleus; A1 = ventrolateral medulla; A5 = ventrolateral pons; SCG = superior cervical ganglion; CG = ciliary ganglion; NE = norepinephrine; Glu = glutamate; GABA = gamma-aminobutyric acid; H = histamine; Ox = orexin; ACh = acetylcholine. Solid lines represent excitatory connections. Dashed lines represent inhibitory connections. Adapted from “Autonomic Pharmacology of α2-adrenoceptors” by E. Szabadi and C. M. Bradshaw, 1996, Journal of Psychopharmacology, 3, p. 11. And “Comparison of Diphenhydramine and Modafinil on Arousal and Autonomic Functions in Healthy Volunteers” by R. H. Hou, R. W. Langley, E. Szabadi, and C. M. Bradshaw, 2007, Journal of Psychopharmacology, 21, p. 574. Copyright 1996 and 2006 by the British Association for Psychopharmacology.
**Tonic Pupillary Responses**

Tonic pupil size has been measured by examining pupillary responses, independent of psychological stimulation, under a range of luminance and distance levels. These measures have been shown to vary across the lifespan and throughout the day in accordance with changes in alertness.

**Age-related changes in pupil size.** Tonic pupil size varies across the lifespan, with the diameter of the pupil gradually increasing from birth to approximately 20 years of age (Kohnen, Zubov, & Kohnen, 2004; Lowenfeld, 1999; MacLachlan & Howland, 2002), and then declining linearly throughout the rest of the lifespan (e.g., Bitsios, Prettyman, & Szabadi, 1996; Boev et al., 2005; Bourne, Smith, & Smith, 1979; Karatekin, Marcus, & Couperus, 2007; Kumnick, 1954, 1956; MacLachlan & Howland, 2002; Schmid, Ceurremans, Luedtke, Wilhelm, & Wilhelm, 2004). This change in tonic pupil size has been attributed to the development and degeneration of the structure of the eye and the neural structures that control pupil size.

Therefore, tonic pupillary responses outside of the relative range for a particular age group could be indicative of atypical development or degenerative processes affecting the eye and/or neural structures controlling pupil size. For example, it has been found that persons with Alzheimer’s disease (AD) have smaller pupil sizes than healthy controls of the same age (e.g., Fotiou, Fountoulakis, Tsolaki, Goulas, & Palikaras, 2000; Hou et al., 2006; Prettyman, Bitsios, & Szabadi, 1997). The typical decline in pupil size with age is mirrored by a decline in the number of cells within the LC (e.g., Baker, Tork, Hornung, & Halasz, 1989; Chan-Palay & Asan, 1989; Manaye, McIntire, Mann, & German, 1995). However, persons with AD have a more profound cell loss within the rostral portion of the LC than healthy age-matched controls (Chan-Palay & Asan, 1989; German et al., 1992; Mareyniuk, Mann, & Yates, 1986). Therefore, it has
been suggested that the cell loss within the LC leads to decreased inhibition of the EW nucleus and thus a smaller tonic pupil size in those with AD (Hou et al., 2006; Prettyman et al., 1997).

**Arousal-related changes in pupil size.** Tonic pupil diameter has also been shown to vary throughout the day with changes in levels of arousal. The tonic diameter of the pupil is largest during the waking state and decreases with levels of arousal (Lowenstein & Loewenfeld, 1961, 1964; Wilhelm et al., 2001), with the smallest pupil size occurring during REM sleep (Lowenfeld, 1999; Yoss, Moyer, & Hollenhorst, 1970). In addition to pupil size being smaller during periods of low arousal, drowsiness is also accompanied by pupillary oscillations that heighten in depth as sleepiness increases (Lowenfeld, 1999; Wilhelm et al., 2001). This tonic change in pupil size with arousal is attributed to alterations in the balance between the sympathetic and parasympathetic divisions of the pupillary system. During periods of high arousal sympathetic activation is increased and the parasympathetic system is inhibited, but during periods of low arousal sympathetic activation and parasympathetic inhibition is decreased (Lowenfeld, 1999).

There are several interrelated neurological structures and neurochemical systems involved in the control of arousal, and alterations in any of these systems could result in changes in levels of arousal and consequently changes in tonic pupil size. The reticular formation contains circuits of neurons (comprised of the medulla, pons, and midbrain) that, when activated by sensory input, send excitatory connections to the cortex via the dorsal (through the thalamus) and ventral (through the hypothalamus, basal ganglia, and basal forebrain) pathways resulting in arousal or alertness (Jones, 2005; Moruzzi & Magoun, 1949). These neuronal systems maintain arousal through the use of several neurotransmitters, NE, histamine, orexin, ACh, 5-HT, DA, and GABA (see Jones, 2005; Stenberg, 2007, for reviews). Of particular importance to the current
study are the NE (A1/A5 and LC) and hypothalamic (PH and LH) systems, as these systems play a major role in the maintenance of arousal, and in the activation of the sympathetic and inhibition of the parasympathetic pupillary system.

The effects of NE on arousal are mediated by A1/A5 nuclei and the LC (e.g., Stenberg, 2007). The A1/A5 nuclei mediate arousal through projections to the hypothalamus and forebrain (e.g., Espana & Berridge, 2006; Stenberg, 2007); while the LC sends diffuse NE projections to the forebrain, brainstem, and spinal cord (e.g., Berridge & Waterhouse, 2003). Of these NE nuclei, the LC is considered the most important for arousal due to its diverse projections and because it contains the largest group of NE neurons in the brain (Dahlstrom & Fuxe, 1964). There are two action levels of the LC-NE system, tonic and phasic activation. Tonic activation indicates the level of spontaneous firing of LC-NE neurons and is related to changing levels of alertness within the sleep-wake cycle; the LC-NE neurons fire most during waking, are reduced during slow-wave sleep, and are almost absent during REM sleep (Aston-Jones & Bloom, 1981). In addition, during the waking state the tonic levels of the LC-NE system indicate the systems readiness to phasically respond; when tonic activity is elevated or low, phasic activation and behavioral performance is decreased, but when tonic activity is at intermediate levels phasic activation is robust and behavioral performance is excellent (e.g., Aston-Jones & Cohen, 2005). Both tonic and phasic pupillary responses have been found to mirror the respective responses of the LC-NE system; when tonic pupil diameter is elevated or low, phasic diameters are reduced, but when tonic diameters are at intermediate levels, there is phasic dilation (Aston-Jones & Cohen, 2005; Rajkowski, Kubiak, Ivanova, & Aston-Jones, 1998; Rajkowski, Kubiak, & Aston-Jones, 1993). In addition, tonic pupil diameters have been shown to be highly correlated with changes in levels of tonic LC-NE discharge rates (Rajkowski et al., 1993, 1998).
The PH and LH mediate the effects of histamine and orexin on arousal, respectively. Both of these neurochemical systems are responsible for the maintenance of waking and send excitatory connections to cortical and brainstem regions (e.g., Jones, 2005; Stenberg, 2007). Similar to the LC-NE system, histaminergic- and orexin-containing neurons fire most during waking, are decreased during slow-wave sleep, and are almost absent during REM sleep (Lee, Hassani, & Jones, 2005; Takahashi, Lin, & Sakai, 2006). Orexin and histamine act to maintain and preserve optimal states of arousal through enhancing the spontaneous firing of LC-NE neurons (Hou et al., 2006; van den Pol et al., 2002) and to a lesser extent through activation of A1/A5 noradrenergic neurons (Baldo, Daniel, Berridge, & Kelley, 2003), and through providing excitatory feedback between the LH and PH (Hou et al., 2006). In contrast, NE tonically inhibits orexin and histamine release indirectly through activation of the \( \alpha_2 \)-receptors on the ventrolateral preoptic nucleus (VLPO), causing a release of GABA onto the PH (Hou et al., 2006; Li & van den Pol, 2005; Samuels, Hou, Langley, Szabadi, & Bradshaw, 2006). In addition, the LH provides dynorphin to the VLPO to inhibit release of GABA (Hou et al., 2006). Thus, the LC provides a negative feedback system indirectly to the hypothalamus that helps to maintain the optimal intermediate levels of tonic LC-NE discharge rates (Li & van den Pol, 2005; van den Pol, 2002) and consequently pupil sizes.

**Phasic Pupillary Responses**

Phasic pupillary responses have long been used to index the amount of cognitive processing or attention that is allocated to a particular task. These responses have been examined in tasks that measure cognitive load, information processing, learning, memory, perception, language processing, and emotional processing (see Andreassi, 2000; Beatty & Lucero-Wagoner, 2000, for reviews). In general, it has been found that phasic pupil size increases and light reflex
amplitude decreases with escalations in task difficulty (e.g., Bradshaw, 1968; Brown et al., 1999; Hess & Polt, 1964; Steinhauer, Condray, & Kasparek, 2000; Steinhauer, Siegle, Condray, & Pless, 2004). However, it has been found that if the task becomes too difficult (as indicated by poor behavioral performance), phasic pupil size begins to decrease (Aston-Jones & Cohen, 2005). In tasks requiring the allocation of short-term memory, phasic pupil size shows a linear increase as each item is presented, then shows a linear decline as each item is recalled (Kahneman & Beatty, 1967). In addition, during these memory tasks, pupil size is larger when familiar items are being recalled (Beatty & Kahneman, 1966).

Stimuli that are salient to a subject have also been found to elicit a larger phasic pupil size and a decrease in light reflex amplitude. Specifically, target stimuli elicit larger pupillary responses than missed target stimuli and non-target stimuli (pupils may decrease to below baseline levels for non-target stimuli; Beatty, 1982), with the amplitude of these dilations decreasing with time on the task (Beatty, 1982; Karatekin et al., 2007; Qiuyuan, Richer, Wagoner, & Beatty, 1985). In addition, phasic dilations to conditioned targets are larger when the stimulus probability is low (Friedman, Hakerem, Sutton, & Fleiss, 1973; Qiuyuan et al., 1985), and when incentives are high (Kahneman & Peavler, 1969). Stimuli that are social in nature, such as human faces, also appear to be salient and cause increased cognitive attention as evidenced by larger phasic pupillary responses. For example, human faces have been shown to elicit a larger phasic pupil size than shape stimuli in infants one to four months of age (Fitzgerald, 1968). In adults, a larger change in pupil size has been observed to upright human faces than to those that are inverted or scrambled, or to macaque monkey faces (Conway, Jones, DeBruine, Little, & Sahraie, 2008). Also, larger pupillary dilations have been found to accompany the presentation of a human face with direct gaze in adult female subjects than those
with indirect gaze (Porter, Hood, Troscianko, & Macrae, 2006). Finally, aversive stimuli have
been shown to elicit a larger phasic dilation than either neutral (Chapman, Oka, Bradshaw,
Jacobson, & Donaldson, 1999; Sterpenich et al., 2006) or positive stimuli (Libby, Lacey, &
Lacey, 1973), and to elicit a decrease in the amplitude of the light reflex response (Bitsios,
a task becomes more salient and requires more cognitive resources or attention, phasic pupil
diameter increases and light reflex amplitude decreases, but when a stimulus is not salient or
does not require as much attention or cognitive resources pupil size begins to decrease and light
reflex amplitude is increased.

**Tonic and Phasic Responses in Autism Spectrum Disorder**

**Tonic Responses**

**Pupillary responses.** To date, three studies have been published examining tonic
pupillary responses in persons with ASD. First, Rubin (1961) examined dark-adapted pupillary
dilations and light-adapted pupillary constrictions in five children with ASD (7 to 12 years of
age) compared to four typically-developing children (7 to 9 years of age). The ASD group was
found to have a significantly slower pupillary constriction rate to light stimulation, along with an
overall smaller dilation during dark adaptation. More recently, Fan, Miles, Takahashi, and Yao
(2009) examined pupillary light reflexes in 24 persons with ASD (7 to 20 years of age) compared
to 44 typically-developing children (6 to 16 years of age). Similar to the Rubin (1961) study,
Fan et al. (2009) found the ASD group to have longer constriction latencies (time to maximum
constriction), and a smaller maximum constriction amplitude in response to light stimulation
than controls. Finally, as reported earlier, Anderson and Colombo (2009) examined the resting
pupil size in seven children with ASD (2 to 5 years of age) compared to mental ($n = 6$) and
chronological age-matched \((n = 9)\) controls. Pupil size was averaged across the presentation of nine blank grey slides presented for an average of 15 s (presented during the Anderson et al., 2006 study). The ASD group was found to have a significantly larger average pupil size, which remained stable across time, than both control groups who did not differ from each other. Thus, the few examinations of tonic pupil size in ASD implicate atypical tonic responding during both resting and reflex conditions in those with the disorder.

**Non-pupillary autonomic responses.** In agreement with the tonic pupillary results presented above, the examination of baseline or resting cardiac, respiratory, electrodermal and skin temperature responses in individuals with ASD are indicative of atypical tonic activation in those with the disorder. Specifically, persons with ASD have been found to have atypical cardiac responses at rest that include an elevated mean HR (Bal et al., 2009; Cohen & Johnson, 1977; Hirstein et al., 2001; Kootz & Cohen, 1981; Kootz, Marinelli, & Cohen, 1982; Ming, Julu, Brimacombe, Connor, & Daniels, 2005), and decreased respiratory sinus arrhythmia (RSA; Bal et al., 2009; Van Hecke et al., 2009), a putative measure of cardiac vagal tone which is regulated by the parasympathetic system (e.g., Porges, 1995). Decreased cardiac sensitivity to baroreflex, as a measure of parasympathetic output, along with increased diastolic and mean arterial blood pressure has also been found in persons with ASD (Ming et al., 2005). In addition to these cardiac responses, increased respiration rates and skin conductance, and lower skin temperature have also been found in those with ASD (Palkovitz & Wiesenfeld, 1980; Zahn, Rumsey, & Van Kammen, 1987). Based on the results of these autonomic investigations, individuals with ASD appear to have higher tonic sympathetic activity as evidenced by mean HR, blood pressure, respiration rates and skin conductance, along with decreases in tonic parasympathetic input as
evidenced by decreased RSA and sensitivity to baroreflex, along with decreased skin temperature.

**Summary and conclusions.** Examinations of tonic pupillary responses, along with non-pupillary autonomic responses during baseline conditions, implicate an altered balance of inhibitory and excitatory activity within the sympathetic and parasympathetic divisions of the autonomic system in ASD. Specifically, the report of a larger resting pupil size (Anderson & Colombo, 2009) along with the slowed light reflex response and less extensive reflex amplitudes in ASD (Fan et al., 2009; Rubin, 1961) suggest increased tonic sympathetic activation, along with decreased parasympathetic input (e.g., Lowenfeld, 1999). While there are only a few studies on tonic pupil size in ASD, the studies presented above examining baseline/resting levels of cardiac, respiratory, skin conductance, and skin temperature responses further implicate this altered balance of autonomic responding in ASD. In addition, the results of these autonomic investigations implicate an altered ANS balance that mirrors that of the tonic pupillary findings (increased sympathetic and decreased parasympathetic responding in ASD).

As stated earlier, tonic pupil size can be indicative of the effects of age and/or tonic arousal. From birth to 20 years of age, tonic pupil size gradually increases and the average value of the tonic pupil diameter during each year of life is similar across healthy individuals (Kohnen et al., 2004; Lowenfeld, 1999; MacLachlan & Howland, 2002), with a similar age-related arc occurring in maximum constriction and dilation reflex amplitudes (Lowenfeld, 1999). In two of the tonic pupillary studies, the ASD group was age-matched with controls to account for these possible age-related differences (Anderson & Colombo, 2009; Fan et al., 2009); in addition, light reflex latency has been found to be independent of age (Fotiou et al., 2007). Therefore, the differences in resting and reflex pupil measures are most likely not attributable to age, but rather
these differences may be attributable to altered levels of tonic arousal. The tonic diameter of the pupil has been shown to vary with changes in levels of alertness, with the resting pupil diameter increasing and reflex responses becoming more extensive and reliable as the individual becomes more alert (Lowenfeld, 1999; Lowenstein & Loewenfeld, 1961, 1964; Wilhelm et al., 2001; Yoss et al., 1970). The larger tonic pupil size, along with less extensive and slowed reflex responses in ASD may therefore be indicative of higher levels of arousal. This could be the result of an increased level of arousal due to the testing environment or atypical functioning of the neurological systems controlling arousal and pupillary responses.

**Phasic Responses**

**Pupillary responses.** Only three studies have examined phasic pupillary responses in persons with ASD. First, van Engeland, Roelofs, Verbaten, and Siangen (1991) examined the pupillary responses of children with ASD (mean age was 9.7 years) compared to children who were typically-developing and children who had either an externalizing (such as conduct disorder) or internalizing disorder (such as avoidant disorder), to a habituation paradigm using black and white abstract stimuli. While all of the children showed a decrease in phasic pupillary responses to the stimuli across habituation trials, there were no differences between the ASD and control groups. Second, as presented earlier, Anderson et al. (2006) examined the pupillary responses of children with ASD (2 to 5 years of age) compared to mental and chronological age-matched controls to face and non-face static pictures. We found the ASD group to have a significant decrease in pupil size from baseline values to human faces, particularly to the internal features of the face, compared to the control groups that showed an increase in pupil size. In addition, we found these differences to be independent of baseline/tonic pupil size (Anderson & Colombo, 2009). Finally, Falck-Ytter (2008) examined the pupillary responses (normalized
according to baseline values) of children with ASD (mean age was 5 years and 2 months) compared to typically-developing controls in response to upright and inverted human faces. The ASD group was found to have a larger pupil size to inverted faces than controls. In addition, the difference in pupil size between the inverted and upright faces was significantly larger for the ASD group than controls, with the ASD group showing a larger pupil size to the presentation of inverted faces than to those that were presented upright. Based on the data from these studies, children with ASD appear to have atypical phasic pupillary responses, which may be unique to human faces and indicative of atypical processing of these stimuli.

**Non-pupillary autonomic responses.** In addition to the pupillary responses presented above, persons with ASD have also been found to have atypical phasic reactions that manifest in non-pupillary autonomic responses such as cardiac, respiratory, and electrodermal responses. In ASD, these atypical autonomic responses have been found during a variety of tasks that include habituation, target detection, and in response to environmental stressors and socially-relevant stimuli. When a stimulus is presented repeatedly, behavioral and autonomic responses to the stimulus typically decrease over repeated presentations (e.g., Colombo, 1995; Richards, 1997). Persons with ASD have failed to show this expected decline in respiratory, cardiac, and skin conductance responses during a habituation paradigm to shapes (Barry & James, 1988; James & Barry, 1980; 1984); although, as reported above, differences were not found when examining pupil size (van Engeland et al., 1991). The lack of cardiac, respiratory and electrodermal habituation has been suggested as an indication of inadequate stimulus processing in ASD (e.g., Barry & James, 1988; James & Barry, 1980; 1984), and studies on cardiac and electrodermal responses to stimuli that are typically salient to a subject provide further support of an autonomic deficit during stimulus intake in individuals with the disorder.
In general during task performance, a decline in cardiac vagal tone, through measurements such as RSA, is typically associated with increased attentional engagement (e.g., Richards & Casey, 1991; Richards & Cronise, 2000; Richards & Turner, 2001), and is generally seen with corresponding increases in autonomic measures of sympathetic activity such as mean HR, blood pressure and electrodermal responses (e.g., Bernston, Cacioppo, & Quigley, 1991; Recordati, 2003). However, increases in sympathetic levels can also indicate aversive or stress-based responses (e.g., Bosch et al., 2009; Eisenberg et al., 1990; Uchino, Cacioppo, Malarkey & Glaser, 1995), with large increases in sympathetic activation leading to less-than-optimal stimulus processing (e.g., Aston-Jones & Cohen, 2005). In persons with ASD, increases in mean HR (Kootz & Cohen, 1981) and estimates of central cardiac vagal tone (Althaus et al., 2004) during non-social target detection tasks have been found. When presented with environmental stressors persons with ASD have been found to have atypical mean HR responses, with one study finding a lower mean HR when presented with stressors such as increases in luminance, noise, and interactions in a classroom environment (Graveling & Brooke, 1978), while a more recent study found a higher mean HR to stressors such as a loud noise, robot and unstructured time in a laboratory environment (Goodwin et al., 2006).

Finally, in accord with the pupillary studies presented above, examination of cardiac and electrodermal responses in individuals with ASD to stimuli with social relevance has also yielded atypical responses. Persons with ASD have been found to have a higher mean HR during social interactions (Kootz et al., 1982; Kootz & Cohen, 1981). In addition, decreased RSA to an unfamiliar person was found in children with ASD, with increased RSA being related to improved social skills (Van Hecke et al., 2009); decreased RSA to unfamiliar persons has been shown to be related to increases in anxiety in typically developing children (Heilman et al.,
2008), thus in this paradigm decreased RSA may indicate a maladaptive response in ASD. Children with ASD have also been found to lack the decline in RSA, found in control groups, during separation from their mothers (Sigman, Dissanayake, Corona, & Espinosa, 2003) and after a display of emotional distress by an experimenter (Corona, Dissanayake, Arbelle, Wellington, & Sigman, 1998), which has been interpreted as a lack of orienting to a psychosocial stressor. Finally, electrodermal responses in persons with ASD have included a lack of skin conductance increase to the presentation of familiar human eyes (Hirstein et al., 2001), and a smaller amplitude during a task requiring emotional judgment of human faces (Hubert, Wicker, Monfardini, & Deruelle, 2009). Thus, these atypical autonomic responses further support the view of impaired stimulus processing in ASD, with the bulk of the findings implicating atypical processing of stimuli that are socially relevant.

**Summary and conclusion.** In general, phasic pupillary responses have been taken to indicate the amount of cognitive resources or attention that is allocated to a particular task (e.g., Andreassi, 2000; Beatty & Lucero-Wagoner, 2000). When interpreted in this manner, the decreased phasic pupillary response to human faces (Anderson et al., 2006) along with a larger pupil response to inverted than upright human faces in ASD (Falck-Ytter, 2008), can be taken to indicate that children with the disorder allocate less cognitive resources or attention to upright human faces than controls. The examination of non-pupillary autonomic responses provides further support that autonomic reactions during stimulus processing may be altered in individuals with ASD. While the direction and interpretation of these non-pupillary autonomic responses vary by the task employed, the results generally agree with the pupillary examinations in implicating maladaptive stimulus processing in ASD, predominantly to stimuli with social relevance.
Conclusion

The results of the pupillary and non-pupillary autonomic investigations presented above indicate that persons with ASD may have an altered ratio of excitatory and inhibitory activity within the sympathetic and parasympathetic divisions of the ANS; this appears to be skewed during both tonic conditions and during phasic activation to salient stimuli such as those that are socially relevant. Of particular importance to the current investigation are the pupillary examinations indicating that individuals with ASD have larger resting and altered reflex responses, suggestive of higher levels of tonic arousal, and consistent with tonic investigations of non-pupillary autonomic responses in ASD. The phasic pupillary investigations reveal smaller phasic pupillary responses to upright human faces in ASD, signifying decreased attentional or cognitive resource allocation to human faces; which is consistent with non-pupillary autonomic investigations in finding atypical phasic responding to stimuli with social relevance in persons with the disorder.

The NE and hypothalamic systems are involved in both sympathetic activation and parasympathetic inhibition of the ANS, and are central to the production of both pupillary and non-pupillary tonic and phasic autonomic reactions; in addition these systems play a major role in the regulation of arousal. Thus, it seems reasonable to examine whether the NE and/or hypothalamic systems play a role in producing atypical pupillary responses in ASD. For example, the pupillary findings in ASD are consistent with elevated levels of tonic LC-NE activity, which result in increased tonic pupil size along with decreases in phasic pupil responses to salient stimuli along with decreased phasic LC-NE activation (Aston-Jones & Cohen, 2005; Rajkowski et al., 1993, 1998). Therefore, it is possible that aberrant activation of the LC-NE system plays a role in the atypical pupillary responses found in ASD. In addition, because the
hypothalamic system directly inhibits the EW nucleus within the pupillary system and enhances the activity of LC-NE and A1/A5 noradrenergic neurons, which respectively provide indirect and direct feedback to the hypothalamic system, it is also possible that the hypothalamic and A1/A5 nuclei may play a role in producing the atypical pupillary responses in ASD.

Potential Neural Mechanisms of Atypical Pupillary Responses in Autism Spectrum Disorder

Both the NE (A1/A5 and LC) and hypothalamic systems play major roles in balancing the ratio of inhibitory and excitatory activity within the sympathetic and parasympathetic divisions of the pupillary system. Furthermore, atypical functioning and/or impairment in either of these systems could affect this balance and result in atypical tonic and phasic pupillary responses. Therefore, the following section will examine functional, structural, and neurochemical evidence of dysfunction in the NE (A1/A5 and LC) and hypothalamic systems in ASD to determine their potential role in producing atypical pupillary responses in this disorder.

Sleep-Wake Cycle Deficits

The NE (A1/A5 and LC) and hypothalamic systems (PH and LH) are part of the reticular formation that is responsible for the regulation of the sleep-wake cycle (Jones, 2005; Moruzzi & Magoun, 1949). Therefore, if impairment were present in either of these systems, sleep-wake cycle deficits would be expected, and in fact, have been reported to be highly prevalent (44% to 86%) in those with ASD (e.g., Liu, Hubbard, Fabes, & Adam, 2006; Richdale & Prior, 1995). Both parent-report and actigraphic data indicate sleep onset delays (Allik, Larsson, & Smedje, 2006a, 2006b; Giannotti et al., 2008; Honomichl et al., 2002; Krakowiak, Goodlin-Jones, Hertz-Picciotto, Croen, & Hansen, 2008; Oyane & Bjorvatn, 2005; Williams, Sears, & Allard, 2004), night waking (Giannotti et al., 2008; Honomichl et al., 2002; Krakowiak et al., 2008; Williams et
al., 2004), and decreased sleep time in those with ASD (Goodlin-Jones, Tang, Liu, & Anders, 2008; Giannotti et al., 2008; Oyane & Bjorvatn, 2005; Schreck, Mulick, & Smith, 2004). Polysomnographic data provide additional support of sleep-wake cycle disturbances in those with the disorder (e.g., Daoust, Limoges, Bolduc, Mottron, & Godbout, 2004; Limoges, Mottron, Bolduc, Berthiaume, & Godbout, 2005). In addition, hours of sleep per night have been shown to be negatively correlated with severity of ASD symptomology (Schreck et al., 2004), with improvements in ASD symptomology being related to improvements in sleep disturbances (Segawa, Katoh, Katoh, & Normura, 1992).

Norepinephrine System

**Structural impairments.** As stated earlier, both the pons and medulla have been investigated using postmortem and neuroimaging technology (see sections on Pontine theory and Inferior olive theory). In postmortem examinations, six out of the 18 brains of persons with ASD that were examined for pontine abnormalities have revealed impairment within this structure. Rodier et al. (1996) found a shortening of the pons in the brain of a 21-year-old female with ASD compared to an 80-year-old male. Bailey et al. (1998) found an atypical tract within the pontine tegmentum in a 4-year-old male with ASD, a widely dispersed LC region in a 24-year-old male with ASD, and loosely grouped LC neurons in a second 24-year-old male with ASD. Weidenheim et al. (2001) found the dorsal raphe, LC, interpeduncular nucleus, and nucleus of the lateral lemniscus of the pons to contain swollen axon terminals in the brain of an 11-year-old female and 20-year-old male with ASD. However, Martchek et al. (2006) examined the LC region in the brains of five adults with ASD, 19 to 54 years of age, and found no differences in total cell count or volume. In MRI examinations, six studies have found the pontine structure to be significantly smaller in persons with ASD compared to controls (Ciesielski et al., 1997; Craig
et al., 2007; Gaffney et al., 1988; Hashimoto et al., 1991, 1993, 1995), while eight of the studies found no volumetric differences within this region (Elia et al., 2000; Garber & Ritvo, 1992; Hardan et al., 2001; Hashimoto et al., 1992, Hashimoto, Tayama, Miyazaki, Murakawa, Shimakawa et al., 1993; Hsu et al., 1991; Kleiman et al., 1992; Piven et al., 1992).

Within the medulla, postmortem examinations have revealed impairment in 11 of the 14 cases with ASD. Bauman and Kemper (1985, 1994) found inferior olive neurons to be small and pale in three adults with ASD (ages 22, 28, and 29) and significantly enlarged in three children with ASD (ages 9, 10, and 12); in addition, in all ASD brains olivary neurons were found to be abnormally distributed along the periphery of this structure. Rodier et al. (1996) found an absence of the superior olive and facial nucleus, and a shortening of the area between the trapezoid body of the medulla and the inferior olive in the ASD brain (21-year-old female). Bailey et al. (1998) found the shape of the inferior olive to be atypical in three males with ASD (ages 4, 20, and 27), and found an enlarged medulla in the 4-year-old, and a slightly flattened medulla with demarcated pyramids in the 20-year-old. Finally, Weidenheim et al. (2001) found swollen axon terminals within the inferior olive and other portions of the medulla in an 11-year old female and 20-year old male with ASD. MRI examinations have also revealed abnormalities within the medulla in persons with ASD. Specifically, four studies have found the medulla to be significantly smaller in persons with ASD (Hashimoto et al., 1992, 1995; Hashimoto, Tayama, Miyazaki, Murakawa, & Kuroda, 1993; Hashimoto, Tayama, Miyazaki, Murakawa, Shimakawa et al., 1993), while two others have found no differences (Gaffney et al., 1988; Hardan et al., 2001).

Summary and conclusion. The most consistent results found from the structural examinations of the medulla and pons in ASD is impairment within the inferior olive of the
medulla. The inferior olive is located within the ventral portion of the medulla, and the ventral lateral (A1) portion of the medulla is part of the NE system that regulates pupil size; thus, impairment within this structure could contribute to altered pupillary responses in ASD.

Impairment within the pontine structure is less conclusive. However, it has been suggested that because the pontine structure has an early development (Bayer et al., 1993) and shows a decline in LC cell number with age (e.g., Baker et al., 1989; Chan-Palay & Asan, 1989; Manaye et al., 1995), that structural abnormalities may only be evident at earlier stages of development and/or in the functional capacity of the NE system (Martchek et al., 2006). Therefore, it is important to conduct further investigations of the pons in young children with ASD to determine its involvement in the disorder. In addition, neurochemical examinations of NE will facilitate further understanding of the function of the NE system in ASD.

**Norepinephrine.** As stated earlier, the LC contains the largest group of NE containing neurons in the CNS (Dahlstrom & Fuxe, 1964) and sends NE innervations throughout the spinal cord, cerebellum, forebrain, cerebral cortex, and hippocampus (see Aston-Jones et al., 1984; Foote et al., 1983 for reviews). The A1/A5 nuclei of the ventral medulla and pons, respectively, also supply a portion of NE innervations, which are sent to the brainstem and hypothalamus (e.g., Kuhar, Couceyro, & Lambert, 1999). Both the LC and A1/A5 nuclei send direct excitatory NE innervations to sympathetic structures controlling pupil size. In addition, both structures are involved in providing inhibitory connections to the EW nucleus of the parasympathetic system; however, while the LC provides direct NE innervations to the EW nucleus, the A1/A5 nuclei provide an indirect influence through the hypothalamic system (Szabadi & Bradshaw, 1996). Therefore, if any of the structures involved in the NE system were impaired in ASD and
involved in the atypical pupillary responses, then alterations in NE levels would also be expected.

Levels of NE in persons with ASD have been examined through blood, urine, and cerebral spinal fluid (CSF). Measurement of plasma levels of NE have yielded the most consistent results, with six studies finding elevated levels of plasma NE in persons with ASD (3- to 23-years-of-age) compared to controls (Cook et al., 1990; Israngkun, Newman, Patel, Duruibe, & Abou-Issa, 1986; Lake, Ziegler, & Murphy, 1977; Launay et al., 1987; Leboyer, Bouvard, & Launay, 1992; Leventhal, Cook, Morford, Ravitz, & Freedman, 1990), and two studies finding no differences (Herault et al., 1994; Martineau et al., 1994). Plasma NE has a short half-life and reflects phasic sympathetic arousal (Minderaa, Anderson, Volkmar, Akkerhuis, & Cohen, 1994). Therefore, the results of these studies suggest that persons with ASD may have atypically heightened sympathetic arousal responses to blood draw (e.g., Cook, 1990; Minderaa et al., 1994). To our knowledge, however, only one study conducted in the last 15 years, has failed to observe differences in the NE system in ASD. This study found no differences between high-functioning adults with ASD and controls in their blood NE responses to a psychosocial stressor (public speaking) (Jansen et al., 2006). Further investigations into task-specific NE responses to a variety of salient stimuli in ASD seem a promising avenue to understand the phasic responding of this system.

In contrast to plasma NE studies, urine and cerebral spinal fluid (CSF) measures of NE reflect time-averaged measures of NE release and are thought to reflect tonic sympathetic arousal. The results of these investigations in ASD have yielded mixed results. Studies examining urine levels of NE and the NE metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) have found increased (Barthelemy et al., 1988; Martineau et al., 1994) and decreased
levels in children (1- to 16-years-of-age) with ASD (Barthelemy et al., 1988; Young, Cohen, Brown, & Caparulo, 1978; Young, Cohen, Caparulo, Brown, & Maas, 1979), while others have shown no differences (Croonenberghs et al., 2000; Launay et al., 1987; Minderaa et al., 1994). Plasma and CSF measures of MHPG have not revealed any differences between persons with ASD (1- to 29-years-of-age) and controls (Gillberg & Svennerholm, 1987; Minderaa et al., 1994; Young et al., 1981). However, because alterations in levels of NE are often not observed until tissue levels are below 90% due to compensatory effects (Abercrombie & Zigmond, 1989), alterations in tonic levels of NE in ASD may only be evident in postsynaptic receptor number and altered levels of second messengers. In fact, all of the studies that have examined the NE second messenger, cyclic AMP (cAMP) have found increases in the plasma (5- to 19-years-of-age) (Goldberg, Hattab, Meir, Ebstein, & Belmaker, 1984; Hoshino et al., 1979, 1980) and CSF levels (Winsberg, Sverd, Castells, Hurwie, & Perel, 1980) in ASD.

**Summary and conclusion.** The relatively consistent finding of heightened levels of plasma NE in response to blood draw suggest that persons with ASD do have atypical phasic sympathetic responses. However, the results of these investigations are limited to phasic responses to only one event, blood draw. Therefore, the NE phasic responses should be further examined in ASD to a variety of salient stimuli and conditions to gain a better understanding of the nature of the NE phasic response impairment in ASD. In addition, future studies should utilize less invasive techniques for examining NE phasic responses, such as saliva measures, to ensure that the responses of the NE system are due to the selected stimulus and not the collection technique. Because the phasic NE investigations in ASD are consistent but limited in number, are limited to responses to blood draw, and are outdated, phasic NE responses in ASD must be reexamined.
While the tonic levels of NE are less conclusive, the consistent finding of elevated levels of cAMP is promising and suggests that impairment to the NE system may only be apparent in compensatory mechanisms. Therefore, tonic NE levels in ASD must also be further examined through measurement of cAMP. In addition, because there have been no studies published to date that have examined NE synthesis through the use of positron emission technology (PET), studies using this technique to examine the regional synthesis of NE levels in ASD is warranted. PET examinations of NE would provide detailed information about the regional differences in NE utilization in ASD and may help to clarify the mixed results of the urine and CSF levels of NE in this disorder.

**Hypothalamic System**

**Structural impairment.** Within the PH and LH, a few postmortem examinations have revealed impairment in persons with ASD. Bauman and Kemper (1994) found the brains of six persons with ASD (9, 10, 12, 22, 28, and 29 years of age) to have reduced neuronal cell size and increased cell number within the mammillary bodies, located on the floor of the PH. Weidenheim et al (2001), found axonal spheroids (swellings of axons) within the mammillary bodies and PH of a 20-year-old male with ASD, and within the LH of both the 20-year-old male and 11-year-old female with ASD. Thus, the most consistent structural impairment within the hypothalamus in persons with ASD is impairment of the mammillary bodies. However, unlike most other hypothalamic nuclei, the mammillary bodies do not relay information to other parts of the hypothalamus and thus are not involved in the regulation of pupil size. Instead, the mammillary bodies act as part of the limbic system relaying impulses from the amygdala and hippocampus to the thalamus. Therefore, these mammillary impairments are more consistent with the limbic system impairments repeatedly found in persons with ASD (see Bauman &
Kemper, 2005; Palmen et al., 2004 for reviews) than impairments within the hypothalamic systems (LH and PH) that help to regulate pupil size.

**Histamine and orexin.** The PH and LH systems that contribute to pupil size and arousal can be investigated by examining levels of histamine and orexin. The tuberomamillary nuclei of the PH are the only source for neuronal histamine in the CNS, and histamine-containing neurons that derive from this nucleus help to maintain arousal and pupil size by enhancing LC-NE neuronal firing and LH activity (Hou et al., 2006). Thus, if atypical functioning of the PH contributes to altered pupillary responses in ASD, atypical levels of histamine would also be expected. However, to my knowledge, only one study has reported an investigation of histamine levels in ASD and no differences between ASD and control groups were found (Launay et al., 1988). Therefore, further investigation into histamine in ASD is necessary to determine if the PH may contribute to atypical pupillary responses in the disorder.

Orexin is a recently discovered neuropeptide, synthesized within the LH (de Lecea et al., 1998; Sakurai et al., 1998), that enhances LC-NE and PH activity (Bayer et al., 2001; van den Pol et al., 2002) to help maintain arousal and pupil size. In addition, orexin-A has been found to modulate activity of the hypothalamic-pituitary-adrenal axis (HPA) by enhancing the release of corticotropin-releasing hormone (CRH) from the paraventricular nucleus (PVN) of the hypothalamus (Russell et al., 2001). The PVN then stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH) into the general circulation resulting in stimulation of the adrenal cortex to release cortisol. Thus, orexin-A indirectly stimulates the release of cortisol through activation of the PVN causing an increase in both ACTH and cortisol (Al-Barazanji, Wilson, Baker, Jessop, & Harbuz, 2001; Ida et al., 2000; Kuru et al., 2000; Mazzocchi, Malendowicz, Gottardo, Aragona, & Nussdorfer, 2001; Spinazzi, Rucinski, Neri, Malendowicz,
& Nussdorfer, 2005). While no studies were found that have examined orexin in persons with ASD, several studies have examined ACTH and cortisol. These studies have yielded mixed results with some finding lower baseline levels of plasma and serum cortisol (Curin et al., 2003; Herman, Arthur-Smith, Hammock, & Josephs, 1988; Hill, Wagner, Shedlarski, & Sears, 1977), and higher baseline levels of urinary cortisol (Richdale & Prior, 1992) and plasma ACTH in ASD (Curin et al., 2003; Tani et al., 2005; Tordjman et al., 1997), while others have found no differences (Brambilla, Viani, & Rossotti, 1969; Goodwin, Cowen, & Goodwin, 1971; Maher, Harper, Macleay, & King, 1975; Sandman, Barron, Chicz-DeMet, & DeMet, 1991; Tordjaman et al., 1997). In addition, studies have revealed abnormal patterns of plasma (Hill et al., 1977; Yamazaki, Saito, Okada, Fujidea, & Yamashita, 1976) and salivary cortisol secretion rhythms in individuals with ASD (Corbett, Mendoza, Abdullah, Wegelin, & Levine, 2006; Corbett, Mendoza, Wegelin, Carmean, & Levine, 2008; Corbett, Schupp, Levine, & Mendoza, 2009; Hoshino et al., 1989). In response to stressors, persons with ASD have been found to have heightened salivary cortisol responses following exposure to a non-social stressor (MRI) compared to controls (Corbett et al., 2006), along with slower salivary cortisol elevation following ACTH stimulation (Marinovic-Curin et al., 2008); while other studies have found no difference in cortisol responses to non-social (mock-MRI) (Corbett et al., 2008, 2009), physical (exercise) or psychosocial (public speaking) stress in persons with ASD (Jansen, Wied, van der Gaag, van Engeland, 2003; Jansen et al., 2006).

**Summary and conclusion.** There is a dearth of structural and neurochemical examinations of the hypothalamic components of the pupillary system (LH and PH) in ASD. Postmortem examinations of the hypothalamus have yielded impairments within the mammillary bodies that are more consistent with limbic system impairments, and which do not indicate LH or
PH impairment in ASD. However, more targeted postmortem and neuroimaging examinations are necessary to determine if the LH and PH are structurally unaltered in ASD. The investigation of histamine and orexin in ASD has been very limited, with one study examining histamine while none have directly examined orexin; therefore, studies directly measuring levels of histamine and orexin in ASD are necessary to determine their involvement in the disorder. However, because orexin-A has been found to modulate the activity of ACTH and cortisol, implications about the responses of the orexin system in ASD can be inferred through examination of cortisol and ACTH levels in ASD. While these examinations have yielded mixed results, it has been suggested that the source of these inconsistencies may be the measurement method. The use of blood draw may lead to altered arousal in persons with ASD, therefore, the results of ACTH and cortisol levels measured through plasma and serum may be indicative of phasic responses to venipuncture (e.g., Jansen et al., 2006; Lam, Aman, & Arnold, 2005). Cortisol measurement through non-invasive techniques, such as saliva, has yielded more consistent tonic results suggestive of impaired secretion rhythms, while phasic salivary cortisol responses to physical and psychological stressors have been less conclusive. Therefore, further investigation into the salivary cortisol levels in ASD is necessary to determine its involvement in the phasic and tonic responses of the hypothalamic system.

**Aim and Predictions of the Current Study**

It is the goal of the current study to replicate and extend the findings from previous investigations (Anderson et al., 2006; Anderson & Colombo, 2009) by examining tonic pupillary responses to a one-minute baseline, and phasic pupillary and scanning responses to social and non-social dynamic and multimodal scenes in young children with ASD. The current study was designed to address some of the methodological concerns of the previous investigation by
including a more homogeneous MA-matched group (e.g., children with Down’s syndrome), and by presenting the children with dynamic and multimodal stimuli that possess more ecological validity than static photos. Finally, we sought to extend previous findings by conducting an examination of the NE and hypothalamic systems through measurement of the salivary correlates of these systems, alpha-amylase (AA) and cortisol, respectively, in response to the social and non-social dynamic and multimodal scenes. The measurement of salivary cortisol and AA in the current study extends previous findings (Anderson et al., 2006; Anderson & Colombo, 2009) by determining the relationship between these salivary measures and phasic and tonic pupillary responses in children with ASD. In addition, the use of these non-invasive salivary measures allowed for the opportunity to determine if the atypical NE and cortisol levels previously found in those with ASD were the result of heightened stress associated with blood draw, given that a measurement of saliva presumably invokes less anxiety.

**Scanning Measures**

The use of static stimuli in eye-tracking investigations has yielded mixed results. Some studies have shown no difference between ASD and control groups scanning of whole upright human faces (Anderson et al., 2006; Speer, Cook, McMahon, & Clark, 2007; Freeth, Chapman, Ropar, & Mitchell, 2009; van der Geest, Kemner, Verbaten, & van Engeland, 2002) or cartoon figures of humans (van der Geest, Kemner, Camfferman, Verbaten, & van Engeland, 2002). Other studies, however, have found persons with ASD to show decreased scanning of whole upright human faces (Riby & Hancock, 2009a; Sasson, Turner-Brown, Holtzclaw, Lam, & Bodfish, 2008), particularly within the eye (Boraston, Corden, Miles, Skuse, & Blakemore, 2008; Dalton et al., 2005; Hernandez et al., 2009; Nacewicz et al., 2006; Neumann, Spezio, Piven, & Adolphs, 2006; Pelphrey et al., 2002; Sterling et al., 2008), nose (Hernandez et al.,
2009; Pelphrey et al., 2002), and mouth (Sterling et al., 2008) region of the faces. In contrast, studies using video clips containing human characters have been consistent in finding scanning differences between ASD and control groups; these studies have found significant decreases in looking time to the eye region (Jones et al., 2008; Klin et al., 2002; Norbury et al., 2009; Riby & Hancock, 2009b; Speer et al., 2007) along with corresponding looking time increases to the mouth (Jones et al., 2008; Klin et al., 2002) and body region of the human characters (Riby & Hancock, 2009b; Speer et al., 2007) in persons with ASD. Thus, it has been suggested that scanning deficits in ASD may be dependent upon the ecological validity of the stimulus (Speer et al., 2007), which has been approximated in these previous studies through the use of social stimuli that is dynamic and multimodal; suggesting that scanning of dynamic social stimuli may be more sensitive to ASD classification than scanning measures to static human faces.

**Hypotheses and predictions.** For the current study, it was predicted that if scanning deficits were present in persons with ASD and sensitive to increased ecological validity, that the ASD group would spend a significantly smaller proportion of time looking at the dynamic social stimulus than either the DS or TD control groups. Specifically, we expected the ASD group to spend a significantly smaller proportion of time looking within the internal feature region than either control group. None of the studies examining scanning of dynamic social stimuli in ASD (Jones et al., 2008; Klin et al., 2002; Norbury et al., 2009; Riby & Hancock, 2008b; Speer et al., 2007) included a non-social dynamic control stimulus. Therefore, the atypical scanning found in individuals with ASD may have been due to the dynamic nature of the stimulus and not the social content per se. In the current study, a non-social dynamic stimulus has been built into the design, which allowed for the determination that differences in scanning were due to the social nature of the stimulus. However, based on the results of a previous investigation (Anderson et
al., 2006), we did not expect any between-group differences in the proportion of time spent looking at the non-social stimulus.

**Pupillary Measures**

The results of previous investigations (Anderson et al., 2006; Anderson & Colombo, 2009) also suggest that children with ASD have a larger tonic (baseline) pupil size, and a smaller phasic pupillary response to human faces than control groups. Studies of tonic pupil size from other laboratories have shown that pupil size varies with sleep-wake cycle changes, with pupil size being largest during states of high arousal or alertness (e.g., Lowenstein & Lowenfeld, 1961, 1964; Willhelm et al., 2001). Therefore, the larger tonic pupil size in children with ASD may be indicative of atypically higher levels of sympathetic arousal (alertness) along with a corresponding decrease in parasympathetic input. This is consistent with tonic pupil (Fan et al., 2009; Rubin, 1961) and non-pupillary autonomic studies (e.g., Bal et al., 2009; Hirstein et al., 2001; Ming et al., 2005; Van Hecke et al., 2009) finding heightened tonic sympathetic responses along with decreased parasympathetic input in ASD, and with studies demonstrating sleep-wake cycle impairments in those with the disorder (e.g., Allik et al., 2006a, 2006b; Giannotti et al., 2008; Honomichl, et al., 2002; Oyane & Bjorvatn, 2005; Williams et al., 2004).

Phasic pupillary responses have been shown to be related to the amount of attention or cognitive resources that are allocated to the processing of information, with the size of the pupil becoming larger as processing demands increase (e.g., Bradshaw, 1968; Steinhauer et al., 2000, 2004) and with increases in social relevance (Conway et al., 2008; Fitzgerald, 1968). Thus, the previous finding of a decreased phasic pupil size to human faces in children with ASD (Anderson et al., 2006) can be taken to indicate less attention or cognitive resource allocation to the processing of stimuli with social relevance in ASD. This finding is further supported by
studies showing atypical phasic pupil (Falck-Ytter, 2008) and non-pupillary autonomic responses (e.g., Corona et al., 1998; Hirstein et al., 2001; Hubert et al., 2009; Kootz et al., 1982; Kootz & Cohen, 1981; Sigman et al., 2003) to stimuli with social relevance in ASD.

**Hypotheses and predictions.** Based on previous pupil and non-pupillary autonomic investigations, it was predicted that the ASD group would have a larger tonic pupil size during the one-minute baseline, consistent with heightened tonic sympathetic activity, than either of the control groups, who were not expected to differ from each other. In addition, if persons with ASD allocate less attention or cognitive resources to stimuli with social relevance, then the results of the current study should be similar to previous pupil and non-pupillary autonomic studies in finding altered phasic pupillary responses to the social stimulus in the ASD group. More specifically, based on previous phasic pupillary studies finding smaller phasic responses to upright human faces (Anderson et al., 2006; Falck-Ytter, 2008), the ASD group was expected to have a smaller phasic pupil response to the social stimulus compared to control groups. Phasic pupillary responses to the non-social stimulus allowed me to determine if these responses were specific to the social stimulus, or if altered phasic pupil size represents a general deficit in attention or cognitive resource allocation. Based on previous investigations (Anderson et al., 2006; van Engeland et al., 1991) no between-group differences in phasic pupillary responses were expected to emerge for the non-social stimulus. No differences in phasic pupillary responses were expected to emerge among the control groups for either stimulus.

**Salivary Measures**

**Alpha-amylase.** The salivary enzyme AA is regulated through NE activation of α- and β-adrenergic receptors, leading to secretion of AA from the parotid salivary gland (Turner & Sugiya, 2002), and thus, salivary AA has been found to vary with changes in plasma levels of
NE (Chatterton, Vogelsong, Lu, Ellman, & Hudgens, 1996; Rohleder, Nater, Wolf, Ehlert, & Kirschbaum, 2004; Wetherell et al., 2006), along with cardiac (Bosch, de Geus, Veerman, Hoogstraten, & Amerongen, 2003; Chatterton et al., 1996; Nater et al., 2006) and electrodermal measures of autonomic activity (El-Sheikh, Erath, Buckhalt, Granger, & Mize, 2008). In addition, like plasma levels of NE, salivary AA decreases in response to β-adrenergic blockers (Speirs, Herring, Cooper, Hardy, & Hind, 1974; van Stegeren, Rohleder, Everaerd, & Wolf, 2006), and increases in response to α2-adrenergic antagonists (Ehlert, Erni, Hebisch, & Nater, 2006). Finally, several studies have found AA to increase in response to psychological stress (Bosch et al., 1996, 1998, 2003; Gordis, Granger, Susman, & Trickett, 2006; Kivlighan & Granger, 2006; Nater et al., 2005, 2006; Rohleder et al., 2004; Rohleder, Wolf, Maldonado, & Kirschbaum, 2006; Skosnik, Chatterton, Swisher, & Park, 2000). Therefore, plasma levels of NE, associated with phasic responses of the NE system, can be estimated through the non-invasive measurement of salivary AA; and to my knowledge salivary AA has not been previously examined in persons with ASD.

**Hypotheses and predictions.** The results of previous investigations showing atypical tonic (Anderson & Colombo, 2009; Fan et al., 2009; Rubin, 1961) and phasic (Anderson et al., 2006; Falck-Ytter, 2008) pupillary responses in persons with ASD, indicate that the functioning of the neurological systems involved in controlling pupil size may be altered in those with the disorder. The findings of structural and neurochemical alterations within the NE system in persons with ASD implicates that the NE system may be a potential source of these altered responses. The atypical resting and reflex tonic pupillary responses along with decreased phasic pupil responses are consistent with elevated levels of tonic NE activity, which result in reduced NE phasic activation, a larger resting pupil size, and reduced phasic pupillary responses to salient
stimuli (e.g., Aston-Jones & Cohen, 2005). The A1/A5 nuclei and LC are part of the pupillary system and their activation results in increased NE sympathetic activity and parasympathetic inhibition of the EW nucleus. Therefore, if the atypical pupillary responses previously found in ASD involve atypical activation of the NE system then, it was predicted that the ASD group would have atypical levels of salivary AA that mirrored their pupillary responses. More specifically, baseline concentrations of salivary AA were expected to be greater for the ASD group compared to controls. In addition, we expected for the ASD groups’ salivary AA concentrations to significantly decrease following the presentation of the social stimulus, while control groups levels were expected to either remain stable or slightly increase. In addition, if the NE system is involved in the atypical pupillary responses in those with ASD, the salivary AA concentrations during both baseline and phasic conditions should be correlated with the corresponding tonic and phasic pupillary responses for the ASD group. No between-group differences in salivary AA responses to the non-social stimulus were predicted.

**Cortisol.** In the current study, salivary cortisol measures were examined as a correlate measure of the hypothalamic system (the HPA system, modulated by the release of orexin-A from the LH). Because both salivary cortisol levels are uncorrelated with salivary AA levels (Chatterton et al., 1996; Granger et al., 2006; Nater et al., 2005, 2006; Nater, Rohleder, Schlotz, Ehlert, & Kirschbaum, 2007) these are not redundant measures. Instead, cortisol and AA are correlates of two independent neurological systems, both of which are responsive to psychological stimuli and are involved in the control of arousal and pupillary responses.

**Hypotheses and predictions.** The hypothalamic system is involved in controlling pupil size and acts in conjunction with the NE system to maintain optimal levels of arousal through sympathetic activation and parasympathetic inhibition. Therefore, if the hypothalamic system is
involved in producing the atypical tonic and phasic pupillary responses previously found in those with ASD, then salivary concentrations of cortisol was also expected to be altered in the ASD group. More specifically, the ASD group was expected to have atypical concentrations of salivary cortisol during both baseline conditions and following the presentation of the social stimulus compared to controls, who were not expected to differ. In addition, if the hypothalamic system is involved in the atypical pupillary responses in those with ASD, then salivary cortisol concentrations during both baseline and phasic conditions were predicted to correlate with the corresponding tonic and phasic pupillary responses for the ASD group. No between-group differences in salivary cortisol responses to the non-social stimulus were expected. Finally, because cortisol responses are uncorrelated with AA responses (Chatterton et al., 1996; Granger et al., 2006; Nater et al., 2005, 2006, 2007), only one of these measures were expected to emerge as a correlate to the tonic and phasic pupillary responses for the ASD group, and no correlations between tonic or phasic concentrations of salivary AA and cortisol levels were anticipated.

Method

Participants

**Inclusion criteria.** Children between the ages of 20 to 72 months of age were recruited for the current study if they had a diagnosis of Autistic Disorder (AD), Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS), Down Syndrome (DS) without a comorbid AD or PDD-NOS diagnosis, or if they were typically-developing (TD). Children were recruited through mail from a variety of developmental disability organizations in metropolitan and suburban areas of Kansas City, KS and MO, and through a pre-established commercial list of families in Johnson, Wyandotte, and Douglas county Kansas. Using these criteria 37 children were recruited for participation and seen at the laboratory for testing sessions.
**Exclusion criteria.** Out of these 37 children, data for 32 of the participants were used in the final analysis (see sections below on attrition and group assignment). Children were excluded from participation if they had comorbid impairments in vision and/or hearing that could significantly impede their ability to see or hear the stimulus (i.e., severe hearing or vision loss), and if they had motor impairments that would interfere with their ability to sit upright without assistance in a car seat. However, children who had vision, hearing, and/or motor impairments that were corrected and did not interfere with data acquisition were included in the final analysis; these conditions included mild amblyopia or “lazy eye” \(n = 5\), corrective lenses for myopia \(n = 4\), ear tubes with no reported hearing loss \(n = 4\), and low muscle tone \(n = 1\). In addition, with the exception of the AD, PDD-NOS, or DS diagnoses, none of the children included in the final analysis had a history of chronic illness or medication use; the only exception to this was seasonal allergies and occasional anti-histamine use \(n = 8\). All children who participated in the current study were healthy (i.e., did not have any symptoms of acute illness such as cold, flu, allergies, etc.) and medication-free at least 48 hours prior to the testing sessions.

**Attrition.** The data from three children were unusable due to inaccurate or insufficient calibration during both testing sessions, and so their data were excluded from the final analysis; one child with DS had poor calibration on only one testing session, which was not included in the final analysis (see section on visual testing session for further explanation of attrition due to calibration). Additionally, two children were excluded because of the group-matching strategy (see sections below on group assignment). Out of the 32 children used in the final analysis, five children had data for only one testing session because of attrition due to participant drop-out. Thus, 26 children had data included in the final analysis for both testing sessions, while six children had data from only one session.
**Group assignment.** Thirty-two participants were assigned to either the Autism Spectrum Disorder (ASD), Down Syndrome (DS), or typically-developing (TD) groups. The ASD group consisted of children who had been previously diagnosed with either AD \((n = 8)\) or PDD-NOS \((n = 4)\). The DS group consisted of children with a diagnosis of DS, without a comorbid ASD diagnosis \((n = 9)\). The TD group was comprised of children who had scores on all subscales of the Mullen Scales of Early Learning (Mullen, 1995) not less than one standard deviation below the test mean, and who did not have a diagnosed developmental disability \((n = 11)\). The presence or absence of an ASD diagnosis was confirmed through administration of the Autism Diagnostic Observation Schedule-Generic (ADOS-G; Lord, Rutter, & DiLavore, 1997).

The age and gender composition of the ASD group dictated the recruitment and formation of the control groups (DS and TD). The control groups were matched with the ASD group on mean age and frequency of each gender. Age was considered a match if between-group differences in mean age were non-significant, and gender was considered a match if between-group gender frequencies were equal. Using these criteria, the DS group was matched with the ASD group on chronological age (CA), \(t (19) = .243, p = .811\), mental age (MA; based on their Early Learning Composite scores on the Mullen), \(t (19) = .359, p = .723\), and was approximately matched on gender. In addition, subscale age equivalents on the Mullen (Visual Reception, Fine Motor, Receptive Language, and Expressive Language) for the DS group did not vary significantly from those of the ASD group (all \(ps > .25\)). The TD group was matched with the ASD group on CA, \(t (21) = -.289, p = .776\), and gender, but not on MA, \(t (21) = -5.095, p < .025\), or Mullen subscale age equivalents (all \(ps < .025\)). There were no differences among the three groups in any of the demographic measures (all \(ps > .05\)). Table 1 presents age, gender, diagnostic, and demographic information for each group.
### Table 1

**Age, Gender, Diagnostic, and Demographic Information for All Groups**

<table>
<thead>
<tr>
<th>Group</th>
<th>ASD 11 male, 1 female</th>
<th>DS 7 male, 2 female</th>
<th>TD 10 male, 1 female</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender distribution</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>n</strong></td>
<td>12</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td><strong>M CAa</strong></td>
<td>50.25</td>
<td>48.67</td>
<td>51.73</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>30 – 69</td>
<td>20 – 73</td>
<td>34 – 69</td>
</tr>
<tr>
<td><strong>Mullen</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Visual Receptionab</td>
<td>36.17</td>
<td>32.22</td>
<td>56.91</td>
</tr>
<tr>
<td>Range</td>
<td>16 – 54</td>
<td>13 – 54</td>
<td>33 – 69</td>
</tr>
<tr>
<td>Fine Motorab</td>
<td>34.42</td>
<td>29.67</td>
<td>52.55</td>
</tr>
<tr>
<td>Range</td>
<td>16 – 53</td>
<td>17 – 49</td>
<td>33 – 68</td>
</tr>
<tr>
<td>Receptive Languageab</td>
<td>31.70</td>
<td>34.63</td>
<td>59.67</td>
</tr>
<tr>
<td>Range</td>
<td>14 – 49</td>
<td>17 – 55</td>
<td>33 – 69</td>
</tr>
<tr>
<td>Expressive Languageab</td>
<td>31.90</td>
<td>31.50</td>
<td>61.89</td>
</tr>
<tr>
<td>Range</td>
<td>10 – 46</td>
<td>17 – 70</td>
<td>39 – 70</td>
</tr>
<tr>
<td>Composite (MA)ab</td>
<td>33.33</td>
<td>31.44</td>
<td>57.43</td>
</tr>
<tr>
<td>Range</td>
<td>14 – 47</td>
<td>16 – 57</td>
<td>35 – 68</td>
</tr>
<tr>
<td><strong>ADOS-G</strong></td>
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<tr>
<td>Social</td>
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<td>0.75</td>
<td>0</td>
</tr>
<tr>
<td>Communication</td>
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</tr>
<tr>
<td>Behavior</td>
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</tr>
<tr>
<td><strong>Demographic measures</strong></td>
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<tr>
<td>Parent education levelc</td>
<td>13.45</td>
<td>14.44</td>
<td>15.78</td>
</tr>
<tr>
<td>Range</td>
<td>8 – 18</td>
<td>9 – 18</td>
<td>12 – 18</td>
</tr>
<tr>
<td>Mother’s ageed</td>
<td>34.25</td>
<td>33.57</td>
<td>38.88</td>
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<tr>
<td>Range</td>
<td>30 – 38</td>
<td>25 – 41</td>
<td>33 – 47</td>
</tr>
<tr>
<td>Father’s ageed</td>
<td>36.75</td>
<td>35.71</td>
<td>40.75</td>
</tr>
<tr>
<td>Range</td>
<td>33 – 41</td>
<td>27 – 45</td>
<td>35 – 49</td>
</tr>
<tr>
<td>Number of siblingsf</td>
<td>1.64</td>
<td>1.89</td>
<td>2.11</td>
</tr>
<tr>
<td>Range</td>
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<td>0 – 6</td>
<td>1 – 6</td>
</tr>
<tr>
<td>Hours in daycaref</td>
<td>7.50</td>
<td>8.22</td>
<td>4.44</td>
</tr>
<tr>
<td>Range</td>
<td>0 – 42</td>
<td>0 – 35</td>
<td>0 – 15</td>
</tr>
</tbody>
</table>

*Note.* ASD = Autism Spectrum Disorder; DS = Down syndrome; TD = typically developing; CA = Chronological age. Mullen = Mullen Scales of Early Learning (Mullen, 1995); Composite = Early Learning Composite; MA = Mental age; ADOG-G = Autism Diagnostic Observation Schedule-Generic (Lord, Rutter, & DiLavore, 1997); Social = Qualitative impairments in reciprocal social interaction; Behavior = Stereotyped behaviors and restricted interests.

*Presented in months.* *Age equivalent score.* *Number of years beyond high school for both parents.* *Presented in years.* *Includes siblings living at home full- and part-time.* *Number of hours per week in center and home-based daycare and preschool.*

### Standardized Tests

Two standardized tests were used for the current study, the ADOS-G (Lord et al., 1997) and the Mullen Scales of Early Learning (Mullen; Mullen, 1995).

**Autism Diagnostic Observation Schedule-Generic (ADOS-G).** The ADOS-G is a semi-structured play observation, with administered activities designed to provide an opportunity
to observe the presence or absence of the core deficits of AD and PDD-NOS (Lord et al., 1989). The administration of this test is concise and it yields three separate scores that reflect core ASD impairments (Communication, Qualitative Impairments in Reciprocal Social Interaction, and Stereotyped Behaviors and Restricted Interests) along with an overall score; each of the scores are used to assist in diagnosing those whose scores fall within the PDD-NOS/AD range. For the current study, this test was administered to all children that were seen for testing appointments, to confirm the diagnosis of AD or PDD-NOS in the ASD group, and to ensure that the children in the DS and TD were appropriately assigned. Thus, the ADOS-G was used to quantify the level of impairment (if any) and confirm group assignment.

**Mullen Scales of Early Learning (Mullen).** The Mullen is a standardized cognitive assessment that allows for flexibility of administration, which has advantages for children with disabilities. This assessment was administered to all children in the current study for the purposes of group MA matching and description of the populations. The Mullen is a standardized test of general cognitive function/developmental status and is comprised of five subscales: Gross Motor, Visual Reception, Fine Motor, Receptive Language, and Expressive Language. Each subscale yields a T-score, percentile rank, and an age equivalent. All subscales, with the exception of the Gross Motor scale, are summarized into an Early Learning Composite score. Therefore, to assist in brevity of administration, participants were administered all subscales except Gross Motor, for the current study.

**Visual Task**

**Stimuli.** For the visual task, all children were presented with one *social* and one *non-social* stimulus that consisted of dynamic and multimodal color video clips. The social stimulus (see Appendix A, for an example) consisted of digitized video clips taken from “The Wiggles”
video series (Wiggles Touring Pty Limited, 1999, 2000, 2001a, 2001b, 2001c, 2002). These images were chosen because they display brief clips of social interactions among the characters, and clips of the characters speaking to the viewing audience. The video clips provided an opportunity for the child to observe a naturalistic social interplay among characters, and to observe characters attempting to socially engage the child by asking questions or revealing information about himself or the scene context; therefore, these scenes mirror social situations that the children may encounter in everyday settings. Clips were chosen from this video series if they had human characters speaking with each other and/or speaking to the viewing audience, and were excluded if the characters were singing, or included non-human characters (i.e., animals, humans dressed as animals, or talking inanimate objects). The video clips that were chosen varied in length from 16 to 166 s, and were edited together to form a 10-minute clip that was cohesive in both content (i.e., speaking was not “cut-off” mid sentence or context) and form (i.e., there were no pauses between clips).

The non-social stimulus (see Appendix B, for an example) consisted of digitized video clips taken from the “Baby Einstein” video series (The Baby Einstein Company, LLC, 1998, 2002a, 2002b, 2002c). These images were chosen because they displayed toys and objects moving to non-voice sounds and instrumental music, and therefore contained movement, sound, and color, but did not include human or animal pictures/noises (i.e., voices or animal sounds). The clips that were chosen varied in length from 6 to 131 s and were edited together to form a cohesive 10-minute video clip (music appeared continuous and there were no pauses between the clips).

Both stimuli were presented on a 40.6 cm computer monitor, which subtended a 21.6° visual angle at the viewing distance. Each stimulus lasted for 10 minutes and was presented
twice for a total of 20 minutes. Average luminance ($M = 3.0$ lx, range: 2.1 – 3.8 lx) and sound level ($M = 60$ dB, range: 50-63 dB; conversational speech is around 60 dB) were held constant. Immediately prior to the presentation of each stimulus, a blank grey slide, matched for luminance (3.0 lx) with the stimulus clips, was presented for three minutes to obtain baseline measures. Each stimulus was presented on a separate day to avoid interference or overlap among the salivary and pupil response measures.

**Eye-tracking apparatus.** Scanning paths and pupillary responses were recorded using an Applied Science Laboratory (ASL) E6 eye-tracking system, Model 504 (Applied Science Laboratory, 2001). The pan/tilt module, which is a component of the ASL E6 eye-tracking system, uses infrared technology to illuminate the eye and telephoto an image of the eye on an eye camera. The E6 control unit then extracts the pupil and reflection of the light source on the cornea and computes both pupil diameter and line of gaze at a sampling rate of 60 Hz.

Use of this system requires calibration to account for individual differences in the distance between the pupil center and corneal reflection, used to calculate line of gaze. A five-point calibration was administered prior to the presentation of baseline and experimental stimuli. Calibration consisted of dynamic and multimodal cartoons that appeared at five calibration points (Appendix C). During calibration and throughout the visual task, a remote control was used to manually move the pan/tilt module to keep the child’s eye centered in the eye monitor. Calibration was considered successful if corneal reflection and pupil thresholds were consistently tracked, and if crosshairs appeared stable and consistent on the eye monitor while the child was looking at each of the five calibration points.

**Visual testing room setup.** The E6 eye-tracking system (ASL, 2001) with the GazeTracker (GT) interface program (Eye-Gaze Response Interface Computer Aid [ERICA],
2001) was set up in a partitioned interior room, divided into experimenter and participant areas. The ASL pan/tilt module was elevated 7.6 cm below the stimulus monitor within the participant area. In addition, a Panasonic video camera was located on top of the stimulus monitor and connected to a RCA television monitor for the purposes of monitoring the child.

Each child was secured in a child-sized car seat using a five-point restraint, to ensure the child’s safety and to minimize movement. The car seat was securely fastened onto a hydraulic chair, which enable adjustment of the child’s eye height to be approximately centered with the mid-point of the stimulus monitor, which was 124.5 cm from the ground and at a visual angle of 21.57º from the stimulus monitor. The pan/tilt module was repositioned by aiming the remote control through a 232.3 cm² opening, located in the middle partition behind the child’s head. A schematic of the testing room setup is presented in Figure 3.
Salivary Sample Collection

Baseline measures of salivary cortisol and AA were taken 1 minute after the start of the baseline slide. Stimulus response measures of salivary AA were taken at 10 and 20 minutes post-stimulus onset, and salivary cortisol measures were taken at 20 minutes post-stimulus onset. The post-stimulus time of sample collection was based on previous salivary AA and cortisol studies in preschool-aged children suggesting a peak change from baseline levels in AA between 10 and 20 minutes post-stimulus onset, and in salivary cortisol at 20 minutes post-stimulus onset (e.g., Davis & Granger, 2009; Fortunato, Dribin, Granger, & Buss, 2008). Salivary samples were collected at each time point using three Sorbettes, small absorbent sponges provided by Salimetrics LLC, which were simultaneously placed under the child’s tongue for approximately 2 minutes to ensure an adequate amount of saliva collection for assay purposes (based on recommendations by Salimetrics LLC). The three Sorbettes for each time point (baseline, 10 minute post-stimulus, and 20 minute post-stimulus), were then placed into one 2 mL cryovial, and immediately frozen at -20º C. All samples were sent to Salimetrics LLC to be assayed.
The diurnal rhythms of AA show a rapid decrease during morning hours and a gradual increase throughout the afternoon (e.g., Jenzano, Brown, & Mauriello, 1987; Nater, Rohleder, Scholtz, Ehlert, Kirschbaum, 2007; Rantonen & Meurman, 2000; Rohleder et al., 2004), and cortisol is highest upon waking with a sharp decrease in the morning, followed by a gradual decline throughout the rest of the day (Davis, Bruce, & Gunnar, 2002; Nater et al., 2007; Watamura, Donzella, Kertes, & Gunnar, 2004). Therefore, all testing was conducted in the afternoon (between 12:00 p.m. and 5:00 p.m.), as salivary levels of AA and cortisol are more stable during this period and less influenced by diurnal variations. In addition, both tests were conducted at the same afternoon time on different days (baseline collection time difference, \( M = 11.35 \) minutes) to ensure that differences in salivary responses to each stimulus type were not due to diurnal variations.

Finally, stressful and physical events, medication use, caffeine consumption equal to or greater than 200 mg, and food consumption can interfere and contaminate salivary measures of both AA and cortisol (see Granger, Kivlighan, el-Sheikh, Gordia, & Stroud, 2007; Hanrahan, McCarthy, Kleiber, Lutendorf, & Tsalkian, 2006, for reviews). Based on the recommendations from AA and cortisol study reviews (Granger et al., 2007; Hanrahan et al., 2006), parents were asked to ensure that their child did not consume any caffeinated products two hours prior, no food or milk (or other protein-enhanced beverage) at least one hour prior, and no liquids at least 10 minutes prior to the testing sessions. The amount of the caffeine consumption reported in the children who ingested caffeine within 2 to 12 hours prior to the testing session (28 % of the sessions, 8 subjects) was estimated between 3 and 15 mg. Testing appointments were scheduled on typical days that were free of stressful or atypical events (e.g., first day of school, birthday party, loss of a favorite toy, car wreck etc.), and parents were asked to reschedule their
appointments if any unusual or stressful event occurred. Parents were also asked keep their child inactive and to ensure that their child did not engage in any exhaustive physical activity (e.g., running, biking, swimming, etc.) at least one hour prior to the testing sessions; participants were reported to last engage in physical activity between 1.5 and 48 hours prior to the testing sessions ($M = 12.63$ hours). As stated earlier, none of the participants had taken any medications (prescription or over-the-counter) within 48 hours of their scheduled testing appointments. Confirmation of compliance with these restrictions was confirmed prior to the start of each session.

**Procedure**

**Recruitment**

Children were recruited for the current study from October 2007 to July 2009. Families were solicited through a variety of developmental disability organizations (Appendix D) that agreed to assist in the recruitment of prospective participants. The organizations were solicited through phone or e-mail by the researcher or student assistants if they had contact with families who met the study inclusion criteria. Once contacted they were informed about the study purpose and were asked to assist in recruitment by either posting the study information in a newsletter or website, or by delivering the recruitment letter (Appendix E) to families of potential participants via postal mail, e-mail, or in person. In addition, family names were also derived from a purchased commercial list, and families from this list were sent the recruitment letter via postal mail, if they had children who could meet the study inclusion criteria.

Once parents received the recruitment letter and determined interest in participation, they contacted our laboratory via telephone or e-mail and were screened for information about the age, diagnosis, comorbid disorders (such as hearing, vision and/or motor impairments), and
current health and medication use for the potential participant. If the child met the study inclusion criteria, then two study appointments were scheduled for the family to come to our Lawrence laboratory. The study appointments were initially scheduled to be approximately one week apart, however, due to rescheduling issues resulting from illness, the occurrence of stressful or unusual events, and other family issues, the actual appointment dates varied between four and 52 days apart ($M = 14.04$ days). Both testing appointments were scheduled to be at the same afternoon time. Parents were informed about food, medication, and physical and stressful event constraints when scheduling their appointments and were reminded of this criterion both verbally and in a written letter (sent via postal or e-mail) during appointment confirmation.

**Data Collection**

As stated earlier, participants were seen at our Lawrence laboratory for two testing sessions that were scheduled at the same afternoon time of day. Only one stimulus was presented at each testing session (either social or non-social), and the order of the stimulus presentation (either session one or two) was counterbalanced.

Upon arrival at the laboratory, participating families were escorted into the standardized testing room where informed consent (Appendix F) was obtained. The parent was then asked to complete the questionnaires in Appendices G and H. The questionnaire in Appendix G was completed during the first testing session and asks about the child’s previous and current health, ASD or DS diagnosis, parent demographic information, medications, food and caffeine consumption, physical activity, and unusual or traumatic events that may have occurred that day. The questionnaire in Appendix H was completed during the second testing session and asks about current health, medications, food and caffeine consumption, physical activity, and unusual or traumatic events that may have occurred that day.
While the parent was completing the questionnaires, a “practice” saliva sample was taken from the child in the standardized testing room to familiarize them with the collection procedure. First, the child was shown the Sorbette, used for sample collection, and was given the opportunity to touch and hold the Sorbette. Next, the collection procedure was described to the child; they were told that they were going to go into another room and watch some videos and that while they were watching these videos the Sorbette was going to be placed under their tongue. Next, the child was asked to open their mouths and the Sorbette was placed under their tongue for approximately 2 minutes; this sample was not tested for either cortisol or AA. Once this procedure and all paperwork were completed, the visual testing procedure began.

**Visual testing procedure.** Parents were invited to accompany their child into the visual testing room during set-up and testing, but siblings were not allowed. A cartoon was playing on the stimulus monitor when the participant entered the visual testing room to help obtain their interest and cooperation with the testing procedure. Once the participant entered the visual testing room, they were shown the apparatus and asked if they would like to sit in the car seat and watch the video. Once assent was received, the child was assisted into the car seat and secured using the five-point restraint to ensure the child’s safety and to minimize movement. Parent(s) who remained in the testing room with the child were asked to either stay in the experimenter area or were allowed to stand behind the child in the participant area. To help ensure that responses were due to the nature of the stimulus and not the ambient environment, neither the parents nor the experimenter interacted with the child or each other during baseline or stimulus conditions.

To begin the visual test, the lights were turned out in the testing room. Then, while the child was watching the cartoon, the child’s left eye\(^1\) was found in the eye camera, and pupil and
corneal reflection thresholds were obtained using the ASL system. A 4 mm model pupil, which is a black aluminum bar with a 4 mm white circle in the center, was then placed over the child’s left eye¹ and the pupil diameter value given by the ASL system was recorded. The recorded value was used to convert the child’s pupil size (obtained in pixels) to mm during data extraction and reduction. Calibration was then completed, and success of calibration was determined by examining pupil and corneal reflection cross hairs at each of the five calibration points. This procedure was repeated up to five times to achieve successful calibration. Once calibration was achieved, either the social or non-social stimulus was presented on the stimulus monitor using the GT program. The child’s behavior was observed via television monitor to ensure safety and compliance during calibration and stimulus presentation.

Each stimulus presentation began with a baseline slide, presented for 3 minutes. At 1 minute after the start of the baseline slide, salivary samples were taken to obtain baseline levels by simultaneously placing three Sorbettes under the child’s tongue for 2 minutes. After the 3 minute baseline slide was presented the experimenter left the participant area and stimulus presentation automatically began. The stimulus was presented using the GT program for 10 minutes. During this time, pupil and scanning data were obtained using the ASL system and eye-tracking was observed on the eye-monitor and ASL system to ensure that the eye remained centered and that pupil and corneal reflection levels were steady and consistent throughout data collection. At 10 minutes post-stimulus onset, a second salivary sample was taken by again placing three Sorbettes under the child’s tongue for two minutes to obtain post-stimulus AA levels. This sample collection coincided with the completion of the 10 minute stimulus presentation, and while samples were collected the scanning and pupil data was saved. The 10 minute stimulus presentation was then repeated using Windows Media Player 9.0 (Microsoft
Corporation, 2004); therefore scanning and pupil responses were not recorded during this time. At 20 minutes post-stimulus onset (recorded from the start of the first 10-minute stimulus presentation), the final salivary sample was taken to obtain post-stimulus levels of AA and cortisol. During salivary sample collection, the experimenter stood beside the car seat in the participant area to obtain samples and did not block the child’s view of the stimulus monitor. Immediately following the collection of each salivary sample (baseline, 10-minute, and 20-minute), the three Sorbettes were placed into one 2 mL cryovial. The cryovials were then labeled with the subject number, sample collection time and date, and stimulus type. Samples were placed into the freezer at the end of the visual testing session. Once the 20-minute saliva sample was taken, the stimulus was turned off and the child was removed from the car seat.

**Standardized testing procedure.** Once the visual task was complete the child was taken back into the standardized testing room to complete either the ADOS-G or the Mullen. The Visual Reception and Fine Motor subscales of the Mullen were completed during the first testing session. During the second testing session, any portion of the Visual Reception or Fine Motor subscales of the Mullen that were not finished during the first session were completed along with the Receptive Language and Expressive Language subscales, concluding Mullen administration. The administration of the ADOS-G followed completion of the Mullen during the second session. Administration of both assessments followed standardization procedures specified in the manuals. After completion of the both tests, scores were sent to the parents in a report letter (Appendix I).

**Data Extraction and Reduction**

**Look zones.** The GT interface program, which was used to present stimulus and baseline slides, extracts line of gaze and pupil size from the ASL E6 eye-tracking system. This program
allows for analysis of scanning and pupil size based on specific areas of interest, and using this program “look zones” were created for each stimulus type. For the non-social stimulus, only one look zone was created for the non-social objects (excluding any blank areas around the objects). See Appendix J for a non-social stimulus example with defined look zones. For the social stimulus, four look zones were created: (a) internal features, which includes the eyes, nose and mouth, (b) external features, which includes the head and neck area and excludes internal features, (c), hands, which includes hands and wrists, (d) body, which includes the torso, arms and legs, and excludes previous zones. See Appendix K for a social stimulus example with defined look zones. The distance of the perimeter of the zone from the actual objects was determined using GT’s recommendations for a five-point calibration, which was 1º visual angle.

**Scanning data.** For the analysis of scanning to each stimulus type, the duration of looking time was examined for the first 10 minutes of stimulus presentation. *Proportion of time tracked* was examined for both the social and non-social stimulus, this variable was calculated as the amount of time tracked across all look zones/ total time tracked on screen. Within the social stimulus, proportion of time tracked for each look zone (internal features, external features, hands, and body) was also examined; this was calculated to be the amount of time tracked within each look zone/ total time tracked on screen.

**Pupillary response data.** Pupil diameter is recorded by the ASL and GT systems at a 60 Hz sampling rate (16.7 ms inter-sampling time). The GT system organizes this data into a time-ordered data file that provides gaze coordinates, pupil diameter in pixels, time of look (from beginning of the video clip), and look zone name in which the gaze occurred. Pupil diameters were converted to mm by dividing the model eye pupil size (4 mm) by the recorded pixel size of the model eye for each child to gain a scale factor; this scale factor was then multiplied by the
recorded pixel value to obtain pupil size in mm at each data point. These data files were then examined and traces of pupil data that occurred on the screen for baseline and stimulus slides were inspected and corrected for artifacts (blinks, loss of tracking, partial eyelid closures, head movements, and accommodation responses) using linear interpolation. Artifacts were identified as (a) a time difference between data points that is greater than 20 ms, (b) a discontinuity in pupil data, or (c) a difference in pupil size that is greater than 0.20 mm. Each pupil trace included in the final analysis was at least 500 ms in length, with artifacts no longer than 500 ms. In addition, if artifacts were present within the trace, the summed duration of these artifacts could be no more than 20% of the duration of the pupil trace.

Once adequate pupil traces were found and corrected for artifacts, an average pupil size was computed for the first minute of the baseline slide. Next, because phasic pupillary responses (but not tonic pupil size) has been found to decrease in amplitude over time on the task (e.g., Beatty, 1982), average pupil size to the first 10 minutes of each stimulus slide was broken into 1-minute epochs. The average pupil size during each time epoch was then subtracted from the average pupil size of the baseline for each stimulus type and within each look zone. These artifact removal and difference score computation methods are similar to those used in several previous studies (e.g., Granholm, Asarnow, Sarkin, & Dykes, 1996; Libbey et al., 1973; Matthews, Middleton, Gilmartin, & Bullimore, 1991; Steinhauer et al., 2004; Verney, Granholm, & Dionisio, 2001).

**Salivary data.** Every three months, salivary samples were mailed on dry ice, via FedEx overnight, to Salimetrics, LLC to be assayed for both cortisol and AA. Once received and tested, Salimetrics, LLC returned the assay results via e-mail in a data file providing cortisol (µg/dL) and AA concentrations (U/mL) for baseline and post-stimulus time points. Because salivary AA
distributions are typically highly variable and positively skewed, salivary concentrations of AA (U/mL) were subjected to square-root transformations based on recommendations from previous studies (e.g., Gordis et al., 2006; Granger et al., 2007). As in previous cortisol studies (e.g., Fortunato et al., 2008) to adjust for positive skew of the cortisol concentrations (ug/dL), a logarithm transformation was completed. Post-stimulus values of cortisol and AA were then subtracted from baseline values to determine the amount of change in cortisol 20 minutes post-stimulus onset and AA at 10 and 20 minutes post-stimulus onset for both social and non-social conditions. Salimetrics, LLC disposed of all samples after assays were completed.

Results

Scanning Measures

We first sought to determine if scanning deficits were present in the ASD group, and if scanning differences were sensitive to the increased ecological validity of the dynamic video clips. To do this, we examined between-group differences in proportion of time tracked for responses within the specified look zones of the social and non-social stimulus for the entire 10-minute presentation period. In addition, to ensure that the results of the scanning analyses were not a function of between-group changes in scanning profiles over the 10-minute presentation period, responses to each stimulus type were split into two time epochs (a) $T1 =$ the amount of time tracked within each look zone during minutes 1 to 5 minutes of stimulus presentation/ total time tracked during minutes 1 to 5 on screen, and (b) $T2 =$ the amount of time tracked within each look zone during minutes 6 to 10 of stimulus presentation/ total time tracked during minutes 6 to 10 on screen. Due to the small sample size, the scanning data were evaluated in two 5-minute epochs (instead of 10 one-minute epochs) to maintain power and ensure that homogeneity-of-variance assumptions were not violated.
Responses to the social stimulus. Repeated measures ANOVA tests were conducted to evaluate between- and within-group differences in proportion of time tracked to the social stimulus look zones (internal features, external features, hands, and body) for the entire presentation period and for each time epoch (T1 and T2). One outlier within the DS group was identified and removed; calibration for this subject was difficult and out-of-range scanning values may have been due to the inaccuracy of eye-tracking for this subject.

Proportion of time tracked for the total presentation period. The Look Zone (4) × Diagnosis (3) repeated measures ANOVA for proportion of time tracked, revealed a significant main effect of Look Zone, $F(1.586, 36.479) = 11.917, p < .001, \eta^2 = .341$. Other terms from the analysis – the main effect for Diagnosis, $F(2, 23) = 1.396, p = .268, \eta^2 = .108$, and the Look Zone × Diagnosis interaction, $F(3.172, 36.479) = 1.051, p = .384, \eta^2 = .084$ – did not attain significance. Follow-up pairwise comparisons of the Look Zone main effect revealed that all subjects spent a significantly larger proportion of time within the internal features look zone than the external features and hands (all $p < .001$), but spent similar proportions within the body look zone ($p = .188$). In addition, a significantly larger proportion of time was spent examining the body look zone than the hands ($p < .001$). The external features and body look zone did not vary significantly ($p = .159$), but the external look zone was tracked significantly more than hands ($p = .011$). Group and total means and standard deviations for proportion of time tracked within each social look zone are presented in Table 2. Thus, while expected within-group differences in scanning were found, no between-group differences emerged. These results are consistent with our previous investigation (Anderson et al., 2006) in finding children with ASD to have scanning profiles that are similar to control groups.

Table 2
Proportion of Time Tracked to the Social Stimulus for each Look Zone

<table>
<thead>
<tr>
<th>Look Zone</th>
<th>All groups</th>
<th>ASD</th>
<th>DS</th>
<th>TD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$N = 26$</td>
<td>$n = 10$</td>
<td>$n = 7$</td>
<td>$n = 9$</td>
</tr>
<tr>
<td>Internal</td>
<td>.24 (.08)</td>
<td>.26 (.10)</td>
<td>.21 (.09)</td>
<td>.24 (.08)</td>
</tr>
<tr>
<td>External</td>
<td>.16 (.05)</td>
<td>.16 (.06)</td>
<td>.19 (.06)</td>
<td>.14 (.04)</td>
</tr>
<tr>
<td>Hands</td>
<td>.12 (.04)</td>
<td>.12 (.04)</td>
<td>.10 (.02)</td>
<td>.14 (.03)</td>
</tr>
<tr>
<td>Body</td>
<td>.20 (.08)</td>
<td>.17 (.06)</td>
<td>.21 (.12)</td>
<td>.22 (.07)</td>
</tr>
</tbody>
</table>

Note. Data are presented in means. Standard deviations are in parentheses. All means and standard deviations represent the total 10-minute presentation period. ASD = Autism Spectrum Disorder; DS = Down Syndrome; TD = Typically developing, Internal = Internal features; External = External features.

Proportion of time tracked by time epoch. A Time (2) × Diagnosis (3) repeated measures ANOVA for proportion of time tracked was conducted for each of the four social look zones. Table 3 shows that the Time main effect was significant for all look zones (all $p < .001$), with the exception of the “hands” look zone, which approached significance. Thus, the proportion of time tracked within the internal and external features look zones decreased significantly from T1 to T2, while the proportion of time tracked within the body look zone increased significantly. No significant main effect of Diagnosis was found for any of the look zones (all $p > .05$).

To further examine the effect of time on the scanning measures, separate Look Zone (4) × Diagnosis (3) repeated measure ANOVAs were conducted separately for T1 and T2. The results of these analyses were similar to the findings for the total presentation period, yielding a significant main effect of Look Zone, T1: $F(1.795, 41.287)^4 = 28.844, p < .001, \eta^2 = .556$ and T2: $F(1.415, 32.540)^4 = 6.890, p = .007, \eta^2 = .231$, with a non-significant Diagnosis main effect, T1: $F(2, 23) = 2.029, p = .154, \eta^2 = .150$ and T2: $F(2, 23) = 1.557, p = .232, \eta^2 = .119$, and nonsignificant Look Zone × Diagnosis interaction, T1: $F(3.590, 41.287)^4 = 1.170, p = .337, \eta^2 = .092$, T2: $F(2.830, 32.540)^4 = .837, p = .478, \eta^2 = .068$. Significant follow-up comparisons.
remained significant (all $ps < .025$) with the exception of the external features and hands during T2, which approached significance (external features > hands, $p = .068$). In addition, the comparison between internal features and body look zones was significant during T1 (internal features > body, $p < .001$), but not during time 2 ($p = .438$), and not across the total presentation period ($p = .188$). Finally, the comparison between external features and body look zones was significant during T2 (external features < body, $p = .030$), but not during T1 ($p = .349$) and not across the total presentation period ($p = .159$). Therefore, while there were some within-group changes in scanning profiles over time, the between-group results remained consistent with the analysis to the entire presentation period in finding no differences. The scanning profiles for the ASD, DS and TD groups were similar for the entire presentation period and across time epochs.
Table 3

*Summary of Repeated Measures Analysis of Time Differences in Proportion of Time Tracked within the Social Look Zones*

<table>
<thead>
<tr>
<th>Look Zones</th>
<th>All groups (N = 26)</th>
<th>Time Main Effects</th>
<th>Diagnosis Main Effects</th>
<th>Time X Diagnosis Main Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1 ( \bar{X} )</td>
<td>( \bar{X} ) (SD)</td>
<td>( F(1,23) )</td>
<td>( p )</td>
</tr>
<tr>
<td>Internal</td>
<td>.31 (.10)</td>
<td>.21 (.10)</td>
<td>61.276</td>
<td>&lt;.001***</td>
</tr>
<tr>
<td>External</td>
<td>.19 (.05)</td>
<td>.16 (.06)</td>
<td>15.986</td>
<td>&lt;.001***</td>
</tr>
<tr>
<td>Hands</td>
<td>.12 (.04)</td>
<td>.13 (.04)</td>
<td>3.986</td>
<td>.058</td>
</tr>
<tr>
<td>Body</td>
<td>.17 (.07)</td>
<td>.23 (.10)</td>
<td>18.806</td>
<td>&lt;.001***</td>
</tr>
</tbody>
</table>

*Note.* T1 = responses during minutes 1 to 5 of stimulus presentation; T2 = responses during minutes 6 to 10 of stimulus presentation; Internal = internal features; External = external features.  
\( ^* \) Data are presented in means, standard deviations are in parentheses.  
\( *** p < .001 \)


**Responses to the non-social stimulus.** A univariate ANOVA\(^6\) was conducted to evaluate between-group differences in proportion of time tracked to the non-social stimulus for the total presentation period. One outlier within the TD group was identified and removed\(^7\). Homogeneity of variance was found to be significant \((p < .001)\); therefore, the Welch’s variance-weighted ANOVA was used. Although the DS group appeared to spend a smaller proportion of time \((M = .89, SD = .10, n = 8)\) tracking the non-social stimulus than either the ASD \((M = .96, SD = .03, n = 12)\) or TD groups \((M = .97, SD = .02, n = 10)\), no significant between-group differences were found \([\text{Welch’s } F(2, 13.856) = 2.821, p = .094, \eta^2 = .288]\).

**Proportion of time tracked by time epoch.** For the time analysis, one outlier within the TD group was identified and removed\(^2\); this outlier was identical to the outlier in the above analysis of non-social scanning responses for the total presentation period. A Time (2) × Diagnosis (3) repeated measures ANOVA\(^3\) for proportion of time tracked revealed non-significant main effects for Time, \(F(1, 27) = 2.777, p = .107, \eta^2 = .093\), and Diagnosis, \(F(2, 27) = .736, p = .488, \eta^2 = .052\). The Time × Diagnosis interaction also failed to attain significance, \(F(2, 27) = 1.053, p = .363, \eta^2 = .072\). Thus, all subjects \((N = 30)\) spent similar proportions of time scanning the non-social stimulus during both T1 \((M = .53, SD = .10)\) and T2 \((M = .47, SD = .10)\). These results indicate that within-group scanning profiles to the non-social stimulus were similar across time, and as expected no significant between-group differences emerged.

**Pupillary Measures**

**Tonic pupil size.** To determine if the previous finding of a larger tonic pupil size in children with ASD was replicable, between-group differences in the average pupil size (in mm) during the first minute of each baseline slide was examined. Three outliers were identified and eliminated \((\text{ASD, } n = 1; \text{TD, } n = 2)\).
Because the measurement of tonic pupil size was recorded across two testing days, within-group differences in the tonic pupil size measurements during day 1 (tonic pupil size obtained during appointment day 1) and day 2 (tonic pupil size obtained during appointment day 2) were first examined to determine if an effect of measurement order was present. The results of the paired-samples $t$ test indicated no significant differences between tonic pupil size recorded during day 1 ($M = 5.11, SD = 1.03$) and day 2 ($M = 5.21, SD = .91$), $t(22) = -.699, p = .492$. Therefore, tonic pupil size measurements from both testing days were averaged together to form one variable. A univariate ANOVA revealed significant between-group differences in tonic pupil size, $F(2, 28) = 6.620, p = .005, \eta^2 = .337$. As can be seen in Figure 4, the ASD group had a significantly larger tonic pupil size ($M = 5.60, SD = 1.02$) than either the DS ($M = 4.19, SD = .93, p = .002$) or TD groups ($M = 4.53, SD = .71, p = .015$), who did not differ significantly from each other ($p = .439$). This finding provides replication of increased tonic pupil size previously reported by Anderson and Colombo (2009) in a different sample.

Figure 4. Average tonic pupil size from both testing days for the Autism Spectrum Disorder (ASD), Down Syndrome (DS) and Typically developing (TD) groups. ** $p < .025$
**Phasic pupillary responses.** To determine if the ASD group had altered phasic pupillary responses that were specific to the social stimulus, between-group differences in the change in average pupil size (from the baseline values for each stimulus type) to specified look zones within the social and non-social stimulus were examined. Because between-group differences in tonic pupil size were found, baseline pupil size was entered as a covariate to ensure that phasic responses were independent and not mediated by altered baseline values. In addition, because phasic pupil responses have been shown to decrease in amplitude over time, change in average pupil size was evaluated in time epochs.

*Responses to the social stimulus.* Repeated measure ANCOVAs\(^3,8\) were conducted to evaluate between- and within-group differences in change in average pupil size to the four social look zones (internal features, external features, hands, and body) for the entire 10-minute presentation period and across time epochs within the social stimulus. Tonic pupil size, measured during the first minute of the social stimulus baseline slide, was entered as a covariate. Two multivariate outliers\(^2\) were identified and removed (ASD, \(n = 1\); TD, \(n = 1\)).

*Phasic pupil responses for the total presentation period.* The Look Zone (4) \(\times\) Diagnosis (3) repeated measures ANCOVA yielded a nonsignificant main effect for Look Zone, \(F(1.927, 40.466)^4 = .097, p = .902, \eta^2 = .005\), but the main effect for Diagnosis, \(F(2, 21) = 6.942, p = .005, \eta^2 = .398\), and the Look Zone \(\times\) Diagnosis interaction, \(F(3.854, 40.466)^4 = 3.854, p = .043, \eta^2 = .208\), attained significance. As can be seen in Figure 5, follow-up analyses\(^5\) of the Diagnosis main effect was attributable to the ASD group increasing their adjusted mean pupil response to the social stimulus (\(M = .581\)). This varied significantly from the TD group, whose adjusted mean decreased (\(M = -.409, p = .001\)). Differences between the ASD and DS groups approached significance (\(p = .054\)), with the DS groups’ adjusted mean pupil response (\(M = .018\)) increasing.
less than those of the ASD group; no significant differences emerged between the DS and TD groups ($p = .134$).

Follow-up analysis of the significant Look Zone $\times$ Diagnosis interaction were conducted by evaluating three repeated measure ANCOVAs for each diagnostic group. The Look Zone main effect was insignificant for both the ASD, $F(2.302, 16.116) = 2.996, p = .072, \eta^2 = .300$, and DS groups, $F(1.119, 7.195) = 1.631, p = .248, \eta^2 = .214$. However, the main effect was significant for the TD group, $F(2.138, 12.826) = 5.056, p = .023, \eta^2 = .457$, whose phasic pupil response to the internal features look zone was significantly larger than those to the external features ($p = .009$) and body look zones ($p = .002$); all other comparisons were insignificant (all $p$s $>.05$). Observed group and total means and standard deviations for change in average pupil size from baseline values within each social look zone are presented in Table 4.

As with our previous investigation (Anderson et al., 2006), significant between-group differences in phasic pupil responses to the social stimulus emerged, however the direction of these differences were divergent. The current findings suggest that the ASD group had a significant increase in their phasic pupil response to all look zones within the social stimulus, while the DS group had a slightly increased response, and the TD groups’ response decreased and presented a distinct profile across the social look zones.
Table 4

Mean Change in Average Pupil Size from Baseline Values to the Social Stimulus for each Look Zone

<table>
<thead>
<tr>
<th>Look Zone</th>
<th>All groups</th>
<th>Group</th>
<th>Group</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 25</td>
<td>ASD</td>
<td>DS</td>
<td>TD</td>
</tr>
<tr>
<td>Internal</td>
<td>.09 (.68)</td>
<td>.47 (.76)</td>
<td>.10 (.45)</td>
<td>-.37 (.54)</td>
</tr>
<tr>
<td>External</td>
<td>.08 (.70)</td>
<td>.49 (.76)</td>
<td>.13 (.46)</td>
<td>-.42 (.55)</td>
</tr>
<tr>
<td>Hands</td>
<td>.08 (.70)</td>
<td>.45 (.73)</td>
<td>.16 (.52)</td>
<td>-.41 (.57)</td>
</tr>
<tr>
<td>Body</td>
<td>.08 (.70)</td>
<td>.48 (.75)</td>
<td>.15 (.49)</td>
<td>-.43 (.55)</td>
</tr>
</tbody>
</table>

Note.  Data are presented in means.  Standard deviations are in parentheses.  All observed means and standard deviations represent the total 10-minute presentation period.  ASD = Autism Spectrum Disorder; DS = Down Syndrome; TD = Typically developing; Internal = Internal features; External = External features.

Figure 5.  Observed mean change in pupil size from baseline values, to each look zone within the social stimulus, for the entire 10-minute presentation period, by diagnostic group.  ASD = Autism Spectrum Disorder; DS = Down Syndrome; TD = Typically developing; Internal = internal features; External = external features.  ** p < .025
**Phasic pupil responses by time epoch.** A Time (4) × Diagnosis (3) repeated measures ANCOVA was initially planned to evaluate the change in average pupil size across time epochs for each of the four social look zones. However, because a significant main effect of look zone was not found in the previous analysis, change in average pupil size from baseline values to each of the four social look zones were averaged together during each 1-minute epoch to form one social pupil size change variable for 5 time epochs: (a) T1; social pupil size change during minutes 1 and 2, (b) T2; social pupil size change during minutes 3 and 4, (c) T3; social pupil size change during minutes 5 and 6, (d) T4; social pupil size change during minutes 7 and 8, and (e) T5; social pupil size change during minutes 9 and 10. Due to the small sample size, the social pupil size change variable was evaluated in five 2-minute epochs, instead of ten 1-minute epochs, to maintain power and ensure homogeneity.

The Time (5) × Diagnosis (3) repeated measures ANCOVA\(^3, 8\) yielded a non-significant main effect of Time, \(F(2.489, 52.264) = 1.599, p = .207, \eta^2 = .071\), a nonsignificant Time × Diagnosis interaction, \(F(4.977, 52.264) = .192, p = .068, \eta^2 = .174\). Similar to the results for the total presentation period, the main effect of Diagnosis was significant, \(F(6, 38) = 6.951, p = .005, \eta^2 = .398\), with the ASD group having a significantly larger adjusted mean (\(M = .583\)) than both the DS (\(M = .008, p = .049\)) and TD groups (\(M = -.404, p = .001\)), who did not differ from each other (\(p = .147\)). See Figure 6 for group observed means and standard errors for social pupil size change across the 5 time epochs (T1 – T5). Although phasic pupil responses have been shown to decrease over time (e.g., Beatty, 1982), no time-related differences were found in the current study. In addition, between-group differences remained, and attained significance for comparisons between the ASD and DS groups.
Figure 6. Observed means and standard deviations of change in average pupil size to the social stimulus from baseline values, for the five time epochs by diagnostic group. ASD = Autism Spectrum Disorder, n = 9; DS = Down Syndrome, n = 8; TD = typically developing, n = 8; T1 = social pupil size change during minutes 1 and 2; T2 = social pupil size change during minutes 3 and 4; T3 = social pupil size change during minutes 5 and 6; T4 = social pupil size change during minutes 7 and 8; T5 = social pupil size change during minutes 9 and 10.

Responses to the non-social stimulus. A univariate ANCOVA\(^6,7,8\) was conducted to examine between-group differences in phasic pupil response to the non-social stimulus for the total presentation period. Significant between-group differences in change in average pupil size to the non-social stimulus were found, \(F(2, 27) = 1.390, p = .008, \eta^2 = .302\), with the TD group having a decreased adjusted mean pupil response to the non-social stimulus (\(M = -.42\)) that varied significantly from the adjusted mean responses of both the ASD (\(M = .27, p = .002\)) and DS groups (\(M = .07, p = .044\)), whose phasic pupil responses increased and did not vary significantly from each other (\(p = .423\))^5.

Phasic pupil responses by time epoch. To be consistent with the evaluation of phasic pupil responses to the social stimulus, time analyses to the non-social stimulus were conducted
across five 2-minute epochs to evaluate non-social pupil size change: (a) T1; minutes 1 and 2, (b) T2; minutes 3 and 4, (c) T3; minutes 5 and 6, (d) T4; minutes 7 and 8, and (e) T5; minutes 9 and 10. Although no outliers were identified\(^2\), 5 subjects were not included in this time-series analysis because of a lack of adequate data during all 5 time epochs (ASD, \(n = 1\); DS, \(n = 3\); TD, \(n = 1\)).

The Time (5) × Diagnosis (3) repeated measure ANCOVA\(^3\,^8\) yielded a non-significant main effect of Time, \(F(2.715, 51.592) = 1.946, p = .139, \eta^2 = .093\). The Diagnosis main effect, \(F(2, 19) = 6.454, p = .007, \eta^2 = .405\), and Time × Diagnosis interactions, \(F(5.431, 51.592) = 2.816, p = .022, \eta^2 = .229\), attained significance. Similar to the findings for the entire presentation period, pairwise comparisons\(^5\) of the Diagnosis main effect revealed the TD group to have a decreased adjusted mean phasic pupil response to the non-social stimulus (\(M = -.744\)), which varied significantly from the adjusted mean response of both the ASD (\(M = .481, p = .004\)) and DS groups (\(M = .373, p = .018\)), who did not differ from each other (\(p = .807\)).

The significant Time by Diagnosis interaction was evaluated through three within-subjects repeated measure ANCOVA’s\(^3\,^8\) for each diagnostic group. The Time main effect was insignificant for both the ASD, \(F(1.595, 11.163) = .296, p = .701, \eta^2 = .041\), and DS groups, \(F(2.311, 6.934) = .566, p = .615, \eta^2 = .159\), but was found to be significant for the TD group, \(F(2.476, 17.335) = 4.515, p = .021, \eta^2 = .392\). For the TD group, phasic responses during T5 were found to be significantly smaller than the phasic responses during T2, T3 and T4 (all \(p < .05\)); all other comparisons were insignificant (all \(p > .05\))\(^5\). See Table 5 for observed group and total means and standard deviations for change in average pupil size from baseline values to the non-social stimulus, for each time epoch and for the entire presentation period.
As with phasic responses to the social stimulus, between-group differences in phasic pupillary responses to the non-social stimulus emerged, but were unique in distinguishing the TD from both clinical groups (ASD and DS); the TD group again showed a decreased response to the stimulus, and ASD and DS groups showed an increased response that did not differ. These between-group differences remained once these phasic responses were evaluated in time-epochs, but increased significantly during T5 for the TD group only.

Table 5

Mean Change in Average Pupil Size from Baseline Values to the Non-Social Stimulus

<table>
<thead>
<tr>
<th>Time Epoch</th>
<th>Group</th>
<th>n</th>
<th>ASD</th>
<th></th>
<th>DS</th>
<th></th>
<th>TD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n = 9</td>
<td></td>
<td>n = 5</td>
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<td>n = 9</td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>.47 (1.03)</td>
<td></td>
<td>.07 (.80)</td>
<td></td>
<td>-.75 (.63)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>.65 (.99)</td>
<td></td>
<td>.25 (.63)</td>
<td></td>
<td>-.90 (.60)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>.81 (1.10)</td>
<td></td>
<td>.96 (.65)</td>
<td></td>
<td>-.75 (.93)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>-.002 (1.15)</td>
<td></td>
<td>.54 (.84)</td>
<td></td>
<td>-.79 (.86)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T5</td>
<td>.04 (1.50)</td>
<td></td>
<td>.43 (.70)</td>
<td></td>
<td>-.31 (.92)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>All Epochs</td>
<td></td>
<td>n = 10</td>
<td></td>
<td>n = 8</td>
<td></td>
<td>n = 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>.20 (.53)</td>
<td></td>
<td>.22 (.33)</td>
<td></td>
<td>-.37 (.33)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note. Data are presented in observed means. Standard deviations are in parentheses. ASD = Autism Spectrum Disorder; DS = Down Syndrome; TD = Typically developing; T1 = non-social pupil size change during minutes 1 and 2; T2 = non-social pupil size change during minutes 3 and 4; T3 = non-social pupil size change during minutes 5 and 6; T4 = non-social pupil size change during minutes 7 and 8; T5 = non-social pupil size change during minutes 9 and 10; All Epochs = non-social pupil size change during the entire 10-minute presentation period.

Salivary Measures

Tonic levels. To determine if the ASD group had atypical baseline levels of AA and/or cortisol, between-group differences in the baseline salivary measures were examined. One ASD subjects’ AA level and another ASD subjects’ (n = 1) cortisol level (for one baseline day) were identified as outliers and removed7. In addition, to determine if tonic measures of AA and/or cortisol were related to tonic pupil size, correlation coefficients were also computed.
Because the tonic measures of AA and cortisol were recorded across two testing days, within-group differences in baseline salivary measurements during *day 1* (baseline values of AA or cortisol during appointment day 1) and *day 2* (baseline values of AA or cortisol during appointment day 2) were first examined to determine if an effect of measurement order was present. The results of the paired-samples *t*-test indicated no significant differences between tonic AA measures recorded during day 1 (*M* = 7.66, *SD* = 2.96) and day 2 (*M* = 7.95, *SD* = 2.66), *t*(24) = -.845, *p* = .406, nor between tonic cortisol measures recorded during days 1 (*M* = -2.47, *SD* = .53) and 2 (*M* = -2.65, *SD* = .49), *t*(23) = 1.650, *p* = .113. Thus, tonic salivary measures from both testing days were averaged together to form one variable for tonic AA and one variable for tonic cortisol. See Figure 7 for group means of salivary AA (U/mL) and cortisol (µg/dL) concentrations.

**Alpha-amylase.** A univariate ANOVA for tonic AA revealed significant between-group differences, *F*(2, 32) = 3.516, *p* = .043, *η*² = .195. The ASD group had a significantly lower baseline level of AA (*M* = 6.44, *SD* = 3.22, *n* = 12) than either the DS (*M* = 8.75, *SD* = 1.61, *n* = 9, *p* = .050) or TD groups (*M* = 9.03, *SD* = 2.34, *n* = 11, *p* = .022), who did not differ (*p* = .806). In addition, a significant negative correlation was found between tonic measures of AA and pupil size, *r*(26) = -.462, *p* = .013. This relationship became non-significant once diagnosis was partialed out, *r*_{ab,c}(25) = -.205, *p* = .296, indicating a mediating effect of diagnosis. These between-group differences in tonic AA levels and significant correlation with tonic pupil size are consistent with predictions of NE system involvement in atypical tonic responses in ASD.

**Cortisol.** A univariate ANOVA for tonic cortisol also revealed significant between-group differences, *F*(2, 32) = 4.645, *p* = .018, *η*² = .243. The DS group had a significantly higher baseline level of cortisol (*M* = -2.20, *SD* = .35, *n* = 9) than either the ASD (*M* = -2.71, SD
= .72, n = 12, p = .034) or TD groups (M = -2.89, SD = .34, n = 11, p = .006), who did not differ (p = .406). The correlation between tonic measures of cortisol and pupil size was non-significant, r(24) = -.102, p = .619, and remained non-significant once diagnosis was partialed r_{ab,c}(23) = -.219, p = .293. These between-group differences in tonic cortisol levels, along with the non-significant relationship with tonic pupil size, are inconsistent with predictions of hypothalamic system involvement in ASD. Instead, these between-group differences indicate unique heightened levels of tonic cortisol for the DS group.

Figure 7. Mean salivary concentrations of alpha-amylase (AA) and cortisol for the Autism Spectrum Disorder (ASD), Down Syndrome (DS), and Typically-developing (TD) groups. ** p < .025; * p < .05

**Phasic responses.** To determine if the ASD group had altered phasic salivary responses, between-group differences in the change in AA and cortisol levels (from baseline values for each stimulus type) to the social and non-social stimulus were examined. Because between-group differences in tonic values of both AA and cortisol were found, tonic salivary levels for each stimulus type were entered as covariates in the respective analyses to ensure that phasic salivary
responses were independent and not mediated by baseline values. Correlation coefficients were also computed to determine if phasic AA and/or cortisol responses to each stimulus type were related to the respective phasic pupillary responses.

**Alpha-amylase.** Repeated measure ANCOVAs\(^3,8\) were conducted to evaluate between and within-group differences in phasic salivary AA responses to the social and non-social stimulus at 10- and 20-minutes post-stimulus onset. Table 6 shows adjusted and observed group means and standard deviations of AA responses to the social and non-social stimulus.

**Responses to the social stimulus.** One multivariate outlier\(^2\) from the ASD group was identified and removed. The Time (2) × Diagnosis (3) repeated measure ANCOVA for phasic AA responses to the social stimulus yielded no significant terms: main effect for Time, \(F(1, 22) = 1.476, p = .237, \eta^2 = .063\), main effect for Diagnosis, \(F(2, 22) = .007, p = .993, \eta^2 = .001\) and the Time × Diagnosis interaction, \(F(2, 22) = .473, p = .629, \eta^2 = .041\).

Correlation coefficients were computed among salivary AA responses to the social stimulus at 10- and 20-minutes post-stimulus onset, and the phasic pupil responses within each of the four social look zones. As can be seen in Table 7, none of the zero-order correlations were significant; in addition, these relationships remained non-significant once diagnosis was partialed out (all \(p s > .05\)). Thus, unlike the tonic AA responses, phasic AA responses to the social stimulus are unable to differentiate the ASD group from controls and are unrelated to phasic pupil responses to the social stimulus, which are unique for the ASD group; these findings are incompatible with the prediction of NE system involvement in atypical phasic pupil responding to social stimuli in ASD.

**Responses to the non-social stimulus.** Two multivariate outliers\(^2\) were identified and removed (ASD, \(n = 1\); TD, \(n = 1\)). The Time by Diagnosis (2 X 3) repeated measure ANCOVA
for phasic AA responses to the non-social stimulus was non-significant for the main effects of Time, $F(1, 25)^4 = .423, p = .521, \eta^2 = .017$, and Diagnosis, $F(2, 25) = 1.634, p = .215, \eta^2 = .116$, and Time by Diagnosis interaction, $F(2, 25)^4 = .514, p = .604, \eta^2 = .040$.

Correlation coefficients were computed among phasic salivary AA responses at 10- and 20-minutes post-stimulus onset and phasic pupil responses to the non-social stimulus. The relationship between phasic AA at 10-minutes post-stimulus onset and phasic pupillary responses to the non-social stimulus was significant, $r(24) = .394, p = .047$; once diagnosis was partialed from the equation this relationship became non-significant, $r_{ab.c}(23) = .260, p = .209$, indicating a mediation effect of diagnosis. The relationship between phasic AA at 20-minutes post-stimulus onset and phasic pupillary responses to the non-social stimulus was non-significant, $r(24) = .140, p = .495$, and remained non-significant once diagnosis was partialed out, $r_{ab.c}(23) = .002, p = .992$. While no between-group differences in non-social phasic AA responses were found, the relationship between 10-minute AA levels and non-social phasic pupil responses, which were distinct for the TD group, do appear to be mediated by diagnosis.
Table 6

*Adjusted and Observed Group Means and Standard Deviations for Salivary Alpha-Amylase Responses*

<table>
<thead>
<tr>
<th>Group</th>
<th>ASD</th>
<th>DS</th>
<th>TD</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Adjusted M</td>
<td>M</td>
<td>SD</td>
</tr>
<tr>
<td>Social</td>
<td>n = 9</td>
<td>n = 8</td>
<td>n = 9</td>
</tr>
<tr>
<td>10-minute</td>
<td>-.33</td>
<td>-.55</td>
<td>1.08</td>
</tr>
<tr>
<td>20-minute</td>
<td>-.85</td>
<td>-.82</td>
<td>1.90</td>
</tr>
</tbody>
</table>

| Non-Social | n = 11       | n = 8       | n = 10      |
| 10-minute  | -.60         | -.35        | 1.26        | -.35         | -.51        | .89         | -1.88        | -2.04       | 2.59        |
| 20-minute  | -1.04        | -.66        | 1.89        | -.66         | -.90        | 1.48        | -1.57        | -1.81       | 2.18        |

*Note.* AA = Alpha-amylase; ASD = Autism Spectrum Disorder; DS = Down Syndrome; TD = Typically developing; $M_{adj}$ = group mean adjusted for baseline AA levels; $M$ = observed group mean; SD = standard deviation.

Table 7

*Relationship between Phasic Pupil Responses, and Salivary Alpha-Amylase Responses to the Social Stimulus*

<table>
<thead>
<tr>
<th>AA</th>
<th>Look Zone</th>
<th>Internal</th>
<th>External</th>
<th>Hands</th>
<th>Body</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$r$</td>
<td>$r_{ab,c}$</td>
<td>$r$</td>
<td>$r_{ab,c}$</td>
</tr>
<tr>
<td>10-minute</td>
<td></td>
<td>.275</td>
<td>.329</td>
<td>.294</td>
<td>.358</td>
</tr>
<tr>
<td>20-minute</td>
<td></td>
<td>.305</td>
<td>.378</td>
<td>.296</td>
<td>.376</td>
</tr>
</tbody>
</table>

*Note.* Both zero-order and partial correlations, holding diagnosis constant, among phasic pupillary responses to each look zone and alpha-amylase (AA) responses to the social stimulus are presented. None of the correlations were significant ($p < .05$). Internal = internal features; External = external features.

For zero-order correlations, df = 22; for partial correlations, df = 21.

**Cortisol.** Univariate ANCOVAs were conducted to evaluate between-group differences in phasic salivary cortisol responses to the social and non-social stimulus at 20-minutes post-stimulus onset. Tonic salivary cortisol level, measured during the respective social or non-social baseline, was entered as a covariate.
Responses to the social stimulus. One outlier\(^7\) from the DS group was identified and removed. The results of the univariate ANCOVA for cortisol responses to the social stimulus was significant, \(F(2, 21) = 7.440, p = .004, \eta^2 = .415\). Follow-up tests\(^5\) indicated that the adjusted mean cortisol response of the TD group, which decreased to the social stimulus (\(M = - .384, SD = .40, n = 9\)), was significantly different from the adjusted mean response of the ASD (\(M = .325, SD = .32, n = 9, p = .001\)) and DS groups (\(M = .105, SD = .47, n = 7, p = .024\)), whose cortisol response increased and did not differ (\(p = .259\)). See Figure 9 for observed group mean cortisol responses to the social stimulus.

Correlation coefficients were computed among salivary cortisol responses to the social stimulus and the phasic pupil responses within each of the four social look zones. As can be seen in Table 8, none of the zero-order correlations were significant. Once diagnosis was held constant, the correlations remained non-significant (all \(ps > .05\)), but the coefficients were notably decreased from zero-order coefficient values, indicating that diagnosis may have a mediation effect on these relationships. Although no differences emerged between the DS and ASD groups, the significant between-group finding and change in correlation coefficients once diagnosis was held constant, suggests the possibility that altered cortisol responses may be involved in social phasic pupil responding for the clinical groups (ASD and DS).
Table 8

*Relationship between Phasic Pupil Responses and Salivary Cortisol Responses to the Social Stimulus*

<table>
<thead>
<tr>
<th>Look Zone</th>
<th>Internal</th>
<th>External</th>
<th>Hands</th>
<th>Body</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>( r )</td>
<td>( r_{ab,c} )</td>
<td>( r )</td>
<td>( r_{ab,c} )</td>
</tr>
<tr>
<td>cortisola</td>
<td>.308</td>
<td>.064</td>
<td>.332</td>
<td>.078</td>
</tr>
</tbody>
</table>

*Note.* Both zero-order and partial correlations, holding diagnosis constant, among phasic pupillary responses to each look zone and cortisol responses to the social stimulus are presented. None of the correlations were significant (\( p \)'s > .05). Internal = internal features; External = external features.

\(^a\) For zero-order correlations, \( df = 22 \); for partial correlations, \( df = 21 \).

**Responses to the non-social stimulus.** Two outliers\(^7\) were identified and removed (ASD, \( n = 1 \); TD, \( n = 1 \)). The univariate ANCOVA revealed non-significant between-group differences in the adjusted mean cortisol responses to the non-social stimulus, \( F(2, 23) = 1.316, \ p = .292, \eta^2 = .122 \), for the ASD (\( M = .10, SD = .46, n = 8 \)), DS (\( M = -.03, SD = .37, n = 7 \)) or TD groups (\( M = -.22, SD = .27, n = 8 \)). See Figure 9 for observed group mean cortisol responses to the non-social stimulus. Correlation coefficients were computed among phasic salivary cortisol and pupil responses to the non-social stimulus. This correlation was non-significant, \( r(24) = -.179, p = .381 \), but became significant once diagnosis was held constant, \( r_{ab,c}(23) = -.458, p = .021 \).

Unlike the social phasic cortisol responses, no between-group differences in non-social phasic cortisol responding emerged. However, the significant change in correlation values once diagnosis was partialed from the equation indicates a possible relationship among non-social phasic cortisol and pupil responses that is independent of clinical diagnosis.
Figure 8. Observed group mean change in salivary cortisol responses, from baseline values, to the social and non-social stimulus. ASD = Autism Spectrum Disorder; DS = Down Syndrome; TD = Typically developing.

Relationship among tonic and phasic salivary measures. Correlation coefficients were computed among tonic salivary measures of AA and cortisol, and phasic salivary AA and cortisol responses to each stimulus type to determine if these salivary responses were indeed providing independent measures, as expected. As can be seen in Table 9, none of the correlation coefficients were found to be significant, and thus tonic and phasic salivary AA and cortisol responses are not redundant measures.
Table 9

Relationship between Tonic and Phasic Salivary Measures of Alpha-Amylase and Cortisol

<table>
<thead>
<tr>
<th></th>
<th>Social AA</th>
<th>Non-Social AA</th>
<th>Tonic AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol Social</td>
<td>0.158 (24)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Cortisol Non-social</td>
<td>--</td>
<td>0.280 (28)</td>
<td>0.155 (28)</td>
</tr>
<tr>
<td>Cortisol Tonic</td>
<td>--</td>
<td>--</td>
<td>-0.176 (28)</td>
</tr>
</tbody>
</table>

Note. Zero-order correlation coefficients are presented in cells, df are presented in parentheses. None of the correlations were significant (p’s >.05). AA = Alpha-amylase.

Classification of Group Membership

The above group-based analyses indicate that tonic pupil size, tonic AA levels, and phasic pupil responses to the social stimulus significantly differentiated the ASD group from both the DS and TD groups. Therefore, a discriminant analysis was conducted to determine whether tonic pupil size and tonic AA levels, measured during both the social and non-social baselines, and change in average pupil size, from baseline values, to the social stimulus (across all look zones and sampling times) could successfully identify the diagnostic groups.

The overall Wilks’s lambda was significant, $\Lambda = .319, \chi^2 (6, N = 22) = 20.573, p = .002$, with a non-significant residual Wilks’s lambda, $\Lambda = .883, \chi^2 (2, N = 22) = 2.233, p = .327$; thus only the first discriminant function will be interpreted. Table 10 presents the within-group correlations between the predictors and the first discriminant function as well as the standardized weights.

The discriminant analysis successfully predicted diagnostic group classification for 77.3% of the participants (kappa coefficient = .658). Additionally, the classification was cross-validated using the “leave-one-out” technique and correctly classified 54.5% of the cases. Thus, classification for the overall sample, based on these three predictor variables, was good.
Specifically, classification of diagnostic group yielded 100% correct classification (71.4% cross-validated) for the ASD group, 75% (37.5% cross-validated) for the DS group, and 57.1% (57.1% cross-validated) for the TD group.

Table 10

*Correlations and Standardized Coefficients of Predictor Variables with the First Discriminant Function*

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Correlation coefficient</th>
<th>Standardized coefficients&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonic AA</td>
<td>-.533</td>
<td>-.651</td>
</tr>
<tr>
<td>Tonic pupil size</td>
<td>.487</td>
<td>.590</td>
</tr>
<tr>
<td>Phasic social pupil response</td>
<td>.448</td>
<td>.817</td>
</tr>
</tbody>
</table>

<sup>a</sup> Standardized canonical discriminant function coefficients.

*Note.* Tonic AA = baseline measures of alpha-amylase (AA) taken across two testing days; Tonic pupil size = baseline measures of pupil size, recorded across two testing days; Phasic social pupil response = average change in pupil size, from baseline values, to the all social look zones for the entire presentation period.

Discussion

The goal of the current study was to replicate and extend the results of previous investigations, which found altered tonic (Anderson & Colombo, 2009) and phasic pupillary responses to human faces in children with ASD, with no group-based differences in visual scanning (Anderson et al., 2006). The NE (A1/A5 and LC) and hypothalamic (LH and PH) systems play a major role in balancing the ratio of inhibitory and excitatory activity within the pupillary system, and thus an altered balance of this ratio could result in the atypical tonic and phasic pupil size that was previously found in ASD. Therefore, to extend the results of previous pupillary studies, and determine if central neural components of the pupillary system are potentially dysfunctional in ASD, salivary measures of AA and cortisol were also examined. As stated earlier, salivary levels of AA have been shown to vary with changes in plasma levels of
NE (e.g., Chatterton et al., 1996; Rohleder et al., 2003; Wetherell et al., 2006), and thus were examined in the current study as a correlate of NE-system activity. In addition, orexin-A is released from the LH and has been found to modulate activity of the HPA by indirectly stimulating the release of cortisol through activation of the PVN (e.g., Al-Barazanji et al., 2001; Spinazzi et al., 2005). Thus, while cortisol does not provide a direct measure of LH or PH activity, alterations in cortisol activity, along with significant relationships with pupil size, may indicate possible dysfunction within the hypothalamic component of the pupillary system, and provide an indication of HPA activity and stress-based responses in ASD. In addition, methodological concerns about the ecological validity of static stimuli, presented during the previous investigation, were addressed by presenting dynamic and multimodal video clips depicting human interactions (“social stimulus”) and toys moving to music (“non-social stimulus”). The current study was also distinct in the inclusion of a clinical MA-match control group (DS), along with a CA-match group (TD), which allowed us to determine if altered responses were specific to the ASD group, or a function of mental- and/or chronological-age.

In the current study, the ASD group was significantly distinguished from both clinical (DS) and TD age-matched controls through (a) a larger tonic pupil size, (b) lower tonic levels of AA, which were significantly related to tonic pupil size and (c) increased phasic pupil responses to the social stimulus. These results provide replication of our previous investigations (Anderson et al., 2006; Anderson & Colombo, 2009) in finding group-based differences among the pupillary measures; in addition the lower AA levels in the ASD group provide a unique result that implicates the possible involvement of the NE-system. Furthermore, the visual scanning results of the current study were similar to our previous investigation (Anderson et al., 2006) in finding no group-based differences, indicating that visual scanning deficits may not be a function
of ecological validity but instead may be due to other methodological differences such as age. However, the use of dynamic and multimodal stimuli in the current study did result in a unique direction of the phasic pupillary responses and could provide information that implicates deficits in the processing of motion-based stimuli.

The current study was also successful in utilizing pupillary measures of both tonic size and phasic responding to social stimuli and tonic salivary levels of AA to correctly classify 100% (71.4% cross-validated) of the ASD group. Therefore, results of the current study, along with previous findings, may provide clues about underlying NE-system pathology in ASD, and the potential of non-invasive measures of pupil size and salivary AA in the early identification and screening of the disorder. The following sections delineate these results by measure (visual scanning, tonic pupil and saliva, and phasic pupil and saliva), and the implications and limitations of each finding are discussed.

**Scanning Responses**

Consistent with our previous investigation (Anderson et al., 2006), no between-group differences in scanning profiles to the social or non-social stimulus emerged for the current study, and scanning profiles across social look zones were similar for the ASD, DS, and TD groups. The results of previous research on visual scanning in ASD have been mixed, but it has been suggested that this ambiguity is the result of the type of social stimulus utilized (Speer et al., 2007). Specifically, studies employing socially-relevant stimuli (human-based) that are *dynamic* have been consistent in finding atypical scanning profiles in persons with ASD (Jones et al., 2008; Klin et al., 2002; Norbury et al., 2009; Riby & Hancock, 2009b, Speer et al., 2007), while paradigms utilizing *static* social stimuli (human faces), including our previous investigation, have resulted in mixed findings (e.g., Anderson et al., 2006; Boraston et al., 2008;
Neumann et al., 2006; Sasson et al., 2008; Speer et al., 2007; Freeth et al., 2009). Therefore, for the current study, we chose to present stimuli that were dynamic and multimodal in nature. We hypothesized a priori that, as with previous studies using this type of stimulus, between-group differences in scanning profiles to the social stimulus would emerge for the ASD group, and vary in degree between the four social look zones (internal features, external features, hands, and body). The non-social stimulus was presented as a “control” stimulus to evaluate if scanning differences were due to the social or dynamic and multimodal nature of the stimulus and no between-group differences were expected to emerge.

While the results of the current study were not consistent with previous investigations using dynamic social stimuli in ASD, this finding is coherent with our previous investigation (Anderson et al., 2006) and others studies that have examined visual scanning to static human faces in children with ASD (Speer et al., 2007; Freeth et al., 2009; van der Geest et al., 2002). Thus, the discrepancy in results between static and dynamic visual scanning investigations may not be due to the ecological validity of the stimulus used, but instead may be a function of other methodological differences such as age. In previous studies (both dynamic and static) where between-group differences emerged, the chronological-age of the participants was slightly older; the majority of these studies included participants that ranged in age from 14 to 35 years of age (Boraston et al., 2008; Dalton et al., 2005; Hernandez et al., 2006; Klin et al., 2002; Nacewicz et al., 2006; Neumann et al., 2006; Norbury et al., 2009; Speer et al., 2007; Sterling et al., 2008), or included a wide age-range of older children and adult participants, 8 to 28 years of age (Ribi & Hancock, 2009a, 2009b; Sasson et al., 2008). The exception is Jones et al. (2008), who found between-group differences among 2-year-old children. In contrast, previous studies with null results have recruited a narrower and younger age-range of children, e.g., 2 to 5 year of age.
This suggests that visual scanning deficits to socially-relevant stimuli in ASD may have a developmental course, with the emergence of more robust and obvious deficits occurring during late childhood or early adolescence, based on the age-range of previous studies finding significant between-group differences. Furthermore, the finding of atypical pupillary responses to social stimuli in the current and previous studies (Anderson et al., 2006; Falck-Ytter, 2008), suggests that the information processing of these stimuli may be altered in young children with ASD. Thus, it is possible that some more fundamental deficit in response or reaction to socially-relevant stimuli in young children with ASD may lead to less adaptive behavioral responses (e.g., decreased visual scanning) later in life.

A second possibility is that scanning differences to socially-relevant stimuli do exist in young children with ASD, but are strongly affected by some non-obvious aspect or property of the stimulus. Thus, previous studies examining orienting to human faces and voices in a naturalistic environment, found decreased overt attentional orienting (i.e., looking) in young children, 6 to 64 months-of-age, with ASD (e.g., Maestro et al., 2002; Osterling & Dawson, 1994; Sweetenham et al., 1998; Zwaigenbaum et al., 2005). It might be argued that the social stimulus employed in the current study may not have adequately approximated the naturalistic environment enough to generate differentiation of the ASD group. Future studies examining visual scanning responses to stimuli varying in their approximation of naturalistic “social” situations, across cross-sectional and/or longitudinal age-groups, will help to illuminate the role of age and ecological validity in differentiating those with ASD.
**Tonic Pupil and Salivary Responses**

As predicted, significant between-group differences in tonic pupil size were found, replicating our previous investigation; the ASD group had a significantly larger tonic pupil size than either the DS or TD groups, who were not appreciably different from each other. In addition, only tonic levels of AA were significantly related to tonic pupil size and distinguished the ASD group from both control groups, suggesting aberrant tonic responding of the NE system in ASD. Unexpectedly, increased salivary cortisol levels distinguished the DS group from the ASD and TD groups, but were unrelated to tonic pupil size; this finding is discussed in terms of stress and risk for chronic illness in DS.

**Tonic responses in ASD.** Previous investigations of autonomic function have found altered pupillary (Fan et al., 2009; Rubin, 1961) and non-pupillary autonomic responses during tonic/resting conditions in persons with ASD (e.g., Bal et al., 2009; Cohen & Johnson, 1977; Hirstein et al., 2001; Kootz & Cohen, 1981; Ming et al., 2005; Van Hecke et al., 2009; Zahn et al., 1987). These atypical responses are indicative of an altered ratio of ANS activity that appears to result from tonic increases in sympathetic activation, along with corresponding decreases in parasympathetic activation in ASD. Consistent with these findings, we recently found children with ASD to have a larger tonic pupil size than age-matched controls (Anderson & Colombo, 2009); thus, the current study replicates the results of our previous investigation. This significant finding was extended in the current study through an examination of baseline measures of salivary AA and cortisol. As predicted, based on previous studies demonstrating the independence of these salivary measures (Chatterton et al., 1996; Granger et al., 2006; Nater et al., 2005, 2006, 2007), no relationship was found among tonic measures of AA and cortisol. Only tonic levels of AA were significantly related to tonic pupil size and this measure...
significantly distinguished the ASD group from both control groups, suggesting aberrant tonic responding of the NE system in ASD.

*Alpha-amylase and pupil size.* Because salivary levels of AA have been shown to vary with changes in plasma levels of NE (Chatterton et al., 1996; Rohleder et al., 2004; Wetherell et al., 2006), the lower levels of salivary AA in the ASD group would appear to indicate decreased tonic levels of NE. However, the larger tonic pupil size found in the current and previous study, along with previous findings consistent with elevated sympathetic activation such as increased non-pupillary ANS responses (e.g., Bal et al., 2009; Ming et al., 2005; Zahn et al., 1987), and increased plasma levels of NE (Cook et al., 1990; Israngkun et al., 1986; Lake et al., 1977; Launay et al., 1987; Leboyer et al., 1992; Leventhal et al., 1990) and time-averaged urine measures of MHPG (Barthelemy et al., 1988; Martineau et al., 1994), necessitates further exploration of this finding.

The apparent discrepancy between these findings may be a function of differences in NE receptor density and type within peripheral targets among pupil and salivary systems. Within the salivary system, sympathetic activation results in a higher concentration of AA, along with decreased and thickened saliva volume, and is primarily mediated by β-adrenergic receptors, particularly the β1 subtype (e.g., Busch, Sterin-Borda, & Borda, 2006; Skov et al., 1988). In contrast, as reported earlier, sympathetic stimulation of α-adrenergic receptors dominate NE-mediated increases in tonic pupil size (e.g., Heal, Prow, Butler, & Buckett, 1995; Koudas et al., 2009). Furthermore, β-adrenergic antagonists, such as propranolol, have been shown to reduce salivary levels of AA (Speirs et al., 1974; van Stegeren et al., 2006) and mean HR (Koudas et al., 2009; van Stegeren et al., 2006), while producing no effects on resting pupil size or blood pressure (e.g., Koudas et al., 2009). This further implicates differential involvement of α- and β-
adrenergic receptors in tonic ANS responding. Therefore, the current finding of an inverse relationship among tonic measures of salivary AA and pupil size (that was mediated by diagnosis) could provide clues about the density and functioning of differential adrenergic receptors in ASD.

Furthermore, altered tonic levels of salivary AA have been found to be related to problem social behaviors and stress in typically-developing children. For example, increased tonic AA levels were found to be associated with more internalizing problems, such as anxiety, depression, fear and worry (El-Sheikh et al., 2008) and poor academic performance in 8-year-old children (Granger et al., 2007), while lower tonic levels of AA were found to be related to decreased approach behavior in children 2 years of age (Fortunato et al., 2008) and increased chronic stress in children, 8 to 18 years of age (Wolf, Nicholls, & Chen, 2008). Thus, while more studies are necessary to establish the relationship among social behaviors and basal AA levels, the current study finding of lower AA in the ASD group is, at face value, coherent with the previous study finding these levels to be associated with decreased social approach. In addition, the larger tonic pupil size, which is indicative of tonic increases in arousal (e.g., Lowenstein & Loewenfeld, 1961, 1964; Wilhelm et al., 2001), may along with the lower AA levels may also be compatible with an increased chronic stress response.

**Cortisol and pupil size.** Basal measurements of cortisol were unable to distinguish the ASD group from the DS and TD groups in the current study. As reported earlier, basal measures of plasma and serum cortisol measurement in ASD has yielded mixed results (e.g., Brambilla et al., 1969; Curin et al., 2003; Goodwin et al., 1971; Herman et al., 1988), with these inconsistencies suggested to be due to the measurement method as venipuncture may lead to increased phasic cortisol levels (Jansen et al., 2006; Lam et al., 2005). Salivary measures of
circadian variations in cortisol have yielded more consistent results and indicate atypical tonic responding in ASD (e.g., Corbett et al., 2006, 2008, 2009; Hoshino et al., 1989), but those previous studies examined changes in salivary cortisol levels at several collection points throughout the day; the current study examined a baseline measure of cortisol collected at only one afternoon time. Thus, it is possible that cortisol alterations in ASD are only present when evaluating secretion rhythms, with unaffected overall tonic levels.

**Summary and conclusion.** The larger tonic pupil size and lower AA levels observed here are consistent with dysfunctional tonic ANS activation in ASD, and the relationship among these tonic measures suggests NE system involvement in producing atypical tonic responding in those with the disorder. Further replication and extension of these results are necessary to determine the veracity of this finding and evaluate both the behavioral and neurological implications of these findings in ASD. Future studies examining pupillary and non-pupillary ANS responses along with time-averaged measures of NE release and tonic levels of AA will help to determine the involvement of the NE system in ASD. In addition, evaluation of intracellular signaling mechanisms characteristic of each adrenergic receptor variety (e.g., second messengers and G-proteins) is necessary to further evaluate the involvement of adrenergic-receptor types in ASD. Finally, while tonic cortisol levels were not able to distinguish the ASD group from controls, and were unrelated to tonic pupil size, future investigations examining circadian variations in salivary cortisol, along with neurochemical measurements of the LH and PH (orexin-A and histamine, respectively) may be necessary to determine the role of the hypothalamic component of the pupillary system in ASD.

**Tonic cortisol and DS.** Unexpectedly, the DS group was distinguished from both the TD and ASD groups by heightened tonic levels of cortisol. Cortisol has been found to increase in
response to stressful stimuli such as venipuncture (McCarthy et al., 2009), challenging cognitive tasks (Spinrad et al., 2009), and in response to the laboratory environment (Jones et al., 2006). Therefore, it is possible that the increased cortisol levels found in the DS group could be attributed to an increased stress-response resulting from exposure to the laboratory environment. Alternatively, there may be reason to expect individuals with DS to have higher cortisol levels due to conditions comorbid with, or secondary to the DS diagnosis. For example, cortisol is a correlate of growth deficiencies due to suppression of growth hormone (GH) (Charmandari, Kino, Souvatzoglou, & Chrousos, 2003; Savage et al., 2002, for reviews), and DS is characterized by deficient growth rates (Cronk et al., 1988) that have been found to be responsive to GH treatment in young children with the disorder (Anneren et al., 1999). In addition, heightened cortisol inhibits thyroid-stimulating hormone (TSH) and is associated with slowed growth, cognitive delays, and impaired cardiac function (see Charmandari et al., 2003, for a review); hypothyroidism and congenital heart defects are commonly found in those with DS (Cohen, 2006; Vis et al., 2009, for reviews). Finally, heightened cortisol levels have also been linked to suppressed immune and inflammatory responses (e.g., Charmandari et al., 2003) that are frequently found in individuals with DS (see Kusters, Verstegen, Gemen, & de Vries, 2009, for a review). As this result was unexpected, we did not collect data on these chronic conditions in our DS sample, but the possibility remains that the heightened levels of cortisol seen in the DS group merely represent an increased risk for the thyroid, cardiac, immune-deficient, and/or autoimmune diseases that are comorbid with DS. Thus, examination of salivary cortisol in DS warrants further investigation to evaluate its usefulness in detecting possible risk for these chronic conditions.
Phasic Pupil and Salivary Responses

As expected, phasic pupil responses to the social stimulus differentiated the ASD group from both the DS and TD groups, but the direction of the phasic response was different from our previous investigation; the ASD group showed a phasic increase to the social stimulus, while the DS group had a slightly increased response and the TD group showed a decreased response. The pattern of these phasic responses were similar for the non-social stimulus, but only differentiated the ASD group from TD controls. The distinct pattern of these phasic pupillary responses may be due to the dynamic nature of the stimuli and these findings are discussed below in terms of motion-processing deficits in ASD. No between-group differences in either phasic AA or cortisol responses to either stimulus were able to distinguish the ASD group from both control groups.

Phasic pupil responses. Previous autonomic investigations have found altered phasic pupil and non-pupillary responses to stimuli with social relevance in ASD (Corona et al., 1998; Falck-Ytter, 2008; Heilman et al., 2008; Hirstein et al., 2001; Hubert et al., 2009; Kootz et al., 1982; Kootz & Cohen, 1981; Sigman et al., 2003; Van Hecke et al., 2009), indicative of altered processing in those with the disorder. Consistent with these findings, we recently found children with ASD to have a decreased pupillary response to static photos of human faces, particularly to the internal features of the face, while age-matched controls showed an increased response and did not differ (Anderson et al., 2006). In our previous investigation, no between-group differences were found for phasic pupillary responses to any of the non-face stimuli. Therefore, we concluded that the decreased phasic pupil response of the ASD group was indicative of decreased attention to human faces. However, the results of the current study which reveal significant between-group differences in phasic pupil responses between the ASD and TD groups
to the non-social stimulus, along with significant differences between the ASD and both control
groups to the social stimulus. Contrary to previous work, this suggests that phasic pupillary
responses may represent a more general processing deficit in ASD accentuated by social content.
In fact, a post-hoc paired-samples $t$ test revealed no significant differences between phasic pupil
responses to the social and non-social stimulus for all groups, $t(22) = -0.584, p = .565$, or when
examined by diagnostic group, ASD [$t(7) = -1.161, p = .284$], DS [$t(6) = .234, p = .823$] and TD
group [$t(7) = -0.074, p = .943$]; indicating that similar phasic pupil responses and consequently
cognitive resources and/or attention were allocated to each stimulus type.

The difference between the phasic pupil direction of the current and previous study may
be due to methodological differences in stimulus parameters and complexity. Thus, studies have
found individuals with ASD to have performance deficits (reaction times, reporting accuracy,
and higher coherence thresholds) on tasks utilizing both socially-relevant (human figure) (Freitag
et al., 2008; Koldewyn, Whitney, & Rivera, 2010) and non-social (random dot arrays) stimuli
that contain movement (Freitag et al., 2008; Koldewyn et al., 2010; Spencer et al., 2000), but
have found intact performance when presented with static stimuli compared to controls
(Koldewyn et al., 2010; Spencer et al., 2000). Furthermore, task performance on motion tasks
has been found to be related to language and cognitive ability (Koldewyn et al., 2010; Takarae,
Luna, Minshew, & Sweeney, 2008), but performance deficits on tasks involving biological
motion (human-based) appear to be more robust to ASD classification as between-group
differences persist even when cognitive ability is controlled (Koldewyn et al., 2010). Thus, it is
possible that deficits in motion processing could have resulted in the ASD groups increased pupil
size, as pupil size tends to increase with task difficulty (e.g., Andreassi, 2000; Beatty & Lucero-
Wagoner, 2000); intact processing of static stimuli in ASD would have required less cognitive
effort and resulted in a smaller pupil size. The similar responses to the non-social stimulus provide further support for this position, as ASD-related deficits in motion processing would also be expected to result in an increase in cognitive resources and consequently pupil size to any dynamic stimulus. In addition, the initial findings that motion processing deficits are a consequence of cognitive and language ability, explains why the DS group, who were matched with the ASD group on MA and CA, would also have an increased phasic response to the stimuli. Thus, the visual dynamics of the stimuli used in the current study create more noise in detection of between-group differences to the social stimulus. Finally, the discrepant findings between the TD groups’ responses to static (increased phasic pupil response) and dynamic (decreased phasic pupil response) stimuli may also be a function of the dynamic nature of the stimuli. Movement has been shown to elicit pupillary constriction (e.g., Sahraie & Barbur, 1997), thus the responses of the TD group in the current study are consistent with this observation.

Delays in the development of the dorsal pathway within the visual system (dorsal occipital and parietal lobes), which has been found to result in altered motion processing in several neurodevelopmental disorders (e.g., Gunn et al., 2002; Klaver et al., 2008; Taylor, Jakobson, Maurer, & Lewis, 2009), may help to explain the direction of the phasic pupil responses. Additionally, the finding that persons with ASD have slowed disengagement to peripheral targets (e.g., Landry & Bryson, 2004; van der Geest, Kemner, Camfferman, Verbaten, & van Engeland, 2001) may further support the involvement of dorsal pathway delays in ASD; however, as presented earlier, the timing of disengagement deficits in ASD (slowed responses to targets that occur at 500 msec or less, and preserved responses when targets occur after 700 msec) may be more consistent with the commonly found cerebellum impairment in those with
the disorder (e.g., Casey et al., 1993; Harris et al., 1999; Townsend et al., 1996, 1999; Townsend & Courchesne, 1994). Thus, while neural delays with the dorsal stream may help to explain the direction of the phasic pupil responses, they do not provide an adequate explanation for the ability of performance-based responses to biological motion (e.g., Freitag et al., 2008; Koldewyn et al., 2010) and phasic pupil responses to both static (Anderson et al., 2006) and dynamic (current study) social stimuli to distinguish individuals with ASD from MA- and CA-matched controls.

**Phasic alpha-amylase.** Salivary levels of AA have been shown to vary with changes in plasma levels of NE (Chatterton et al., 1996; Rohleder et al., 2004; Wetherell et al., 2006), indicative of phasic NE system activation (Minderaa et al., 1994). Therefore, because heightened plasma levels of NE have been found in ASD (e.g., Cook et al., 1990; Israngkun et al., 1986; Lake et al., 1977; Launay et al., 1987; Leboyer et al., 1992; Leventhal et al., 1990), phasic AA responses were expected to differentiate the ASD group from controls in current study. In addition, because the NE system plays a central role in balancing the ratio of inhibitory and excitatory activity within the pupillary system, a significant relationship was also anticipated between phasic AA and pupillary responses. AA responses to both the social and non-social stimulus decreased from baseline values for all groups. However in contrast to plasma NE studies in ASD, no between-group differences emerged for the current study. In addition, correlations among phasic AA levels and phasic pupil responses were all insignificant, with the exception of the correlation between 10-minute AA responses and phasic pupil responses to the non-social stimulus. As presented above, non-social phasic pupil responses distinguished the TD group from both clinical groups (ASD and DS) and thus were attributed to developmental delay. Similarly, the correlation between these phasic non-social measures were found to be mediated...
by diagnostic group, thus, it is possible that the relationship between phasic non-social responses may also be a function of differences between the mental age of the TD and clinical groups.

**Phasic cortisol.** As reported earlier, previous investigations of phasic cortisol responses to physical, social, and non-social stressors in ASD have yielded mixed results (e.g., Corbett et al., 2006, 2008, 2009; Jansen et al., 2003, 2006; Marinovic-Curin et al., 2008). These inconsistent findings coupled with the lack of structural abnormalities within the LH or PH in ASD have led to some uncertainty about hypothalamic (LH and PH) and/or cortisol involvement in ASD. Between-group differences did emerge for the current study; phasic cortisol decreased to the social stimulus for the TD group, but was significantly increased for both clinical groups (ASD and DS) who did not differ from one another. Cortisol responses to the non-social stimulus decreased for the TD group, marginally decreased for the DS group, and increased for the ASD group, but these between-group differences did not attain conventional levels of statistical significance. In addition, none of the correlations among phasic pupil and cortisol responses to either stimulus were significant, but coefficients did change once diagnosis was held constant. Thus, coefficients for responses to the social stimulus were notably decreased once diagnosis was partialed out but remained insignificant, while the partial correlation coefficient for responses to the non-social stimulus attained significance. Since the ASD and DS groups did not differ, these results are consistent with the possibility that phasic cortisol responses to the social stimulus (and possibly their relation to phasic pupil responses) are due to differences in MA. It is possible that increased cortisol responses could reflect a heightened level of stress resulting from the presentation of the social stimulus. While this would provide additional clarification of the increased phasic pupil response, this interpretation is only speculative as no
relationship emerged among these phasic measures and does not provide an adequate explanation for increased phasic pupil size that also occurred in the clinical groups to the non-social stimulus.

**Summary and conclusion.** Increased phasic pupillary responses to the social stimulus were able to differentiate the ASD group from both control groups and are indicative of increased attention or cognitive resource allocation that may result from general deficits in motion-processing that are most robust to ASD classification when stimuli with biological motion (human-based) are utilized. Thus, the increased pupil size for both clinical groups to the non-social stimulus further supports the indication of a general motion-processing deficit in developmental disorders and emphasizes the importance of including a non-social “control” stimulus in future studies examining human-based processing in ASD. While the discrepant direction of the phasic pupillary responses, between the current and previous investigation, seem to be logically explained by the dynamic nature of the stimuli, future studies of phasic pupil responding in ASD that integrate both static and dynamic human-based and control stimuli are warranted.

None of the *phasic* salivary correlates distinguished the ASD group from both control groups. However, the altered cortisol response to the social stimulus for both clinical groups indicates a possible developmental effect that may be a function of a stress-based response or delayed development of neural system that underlie and mediate cortisol release. Thus, future neurochemical investigations, measuring NE, cortisol, and LH and PH activation through a variety of mediums (e.g., saliva, plasma, urine, etc.), and phasic pupillary responses in ASD to social and non-social stimuli with differing parameters and complexity are necessary to exclude involvement of these systems in ASD phasic pupil responding.
Conclusions and Limitations

The current study demonstrated that tonic pupil size, tonic AA responses, and phasic pupil responses to the social stimulus were able to significantly differentiate the ASD group from both the DS and TD age-matched controls; and a discriminant analysis indicated that these measures correctly classified 100% (71.4% cross-validated) of the ASD group. The results of the current study are consistent with our previous investigations in finding that both tonic pupil size and phasic pupil responses to a socially-relevant stimulus can distinguish those with ASD from age-matched controls. In addition, the current results extend our previous pupillary findings by indicating that alterations in the NE system may underlie the atypical tonic pupil responses in ASD, and that the direction of the phasic pupil response may be altered by movement.

Pupil size has been shown to change in response to socially-relevant stimuli as early as one month of age (Fitzgerald, 1968). Therefore because both tonic and phasic pupil measures can be obtained during infancy and have been found to significantly distinguish young children with ASD in the current and previous studies, the candidacy of these responses as early indicators of ASD are warranted. The time course for the emergence of salivary AA is more protracted than pupil measures; AA is not present in the saliva until approximately 1 year of age (e.g., Granger et al., 2007). However, the current finding of lower levels of salivary AA that can distinguish young children with ASD from controls merits the investigation of this salivary measure as a potential biological marker of ASD. In addition, because the NE system (A1/A5 and LC) begins neurogenesis very early during the prenatal period (Bayer et al., 1993) and makes vast NE innervations that help to regulate CNS development (e.g., Coyle, 1977; Schlumpf et al., 1980; Sievers et al., 1981), a neurochemical or structural indication of disruption in this
system is likely present during infancy and possibly prenatally. Therefore, because salivary AA was related to tonic pupil size, it could be argued that if tonic pupil size is able to differentiate ASD during infancy, that other correlates of the NE system (neurochemical and structural measures) may also be present during this period and have the ability to identify those with the disorder. In addition, if the NE system is indeed impaired in ASD, measurements of this system during the infancy may lead to a greater understanding of how the pathology and functional impairment of the NE system is involved in producing ASD symptomology.

However, there are several limitations that should be addressed in future investigations. In the current study, the groups were mean-matched on both MA and CA to ensure that group differences were not a function of variations in developmental level. Because we only examined pupil and salivary responses in young children with ASD, future investigations with older children and adults are necessary to determine if the significant findings in the current and previous studies vary across development. Second, some autonomic responses such as blood pressure, respiration rate, and HR have been found to be correlated with body mass index (BMI) (e.g., Gelber, Pfeifer, Dawson, & Shumer, 1997; Nagai & Moritani, 2004; Pitzalis et al., 2000). However, no such responses have been found with pupillary responses (e.g., Filipe, Falcao-Reis, Castro-Correia, & Barros, 2003; Phia, Rommema, & Koskenvuo, 1994) and thus BMI was not examined in the current study. However, BMI has been found to be associated with salivary cortisol responses (e.g., Charmandari et al., 2003), and the BMI of both children with DS (Cronk et al., 1988) and ASD (e.g., Mraz, Green, Dumont-Matbieu, Makin, & Fein, 2007; Torrey, Dhavale, Lawlor, & Yolken, 2004) have been found to be increased. Thus, it is possible that the increased tonic cortisol responses in the DS group, and heightened phasic cortisol reactions to social stimuli in both clinical groups may have been the result of altered BMI; therefore, BMI
should be examined in future pupil and salivary studies. Finally, in the current study, only indirect peripheral measurements (salivary AA and cortisol) were obtained; therefore, any inferences to the functioning of the neurological components of the pupillary system should be interpreted with caution and were included in the current study to determine if further investigation of these pupillary systems in ASD were warranted. Thus, future structural and neurochemical studies are necessary to determine the involvement of the NE system, HPA, and LH and PH in ASD, in addition to examining the candidacy of these responses as early identifiers of the disorder.
Footnotes

1. The exception to this was the participants who had amblyopia of the left eye ($n = 5$) whose right eye was tracked.

2. Multivariate outliers were identified by evaluating Mahalanobis distance at $p < .005$.

3. Homogeneity of the variance-covariance matrices was evaluated at the $p < .001$ level using Box’s M estimate for multivariate tests, and Levene’s estimate at the $p < .05$ level for univariate tests; these assumptions were met unless noted.

4. Due to small sample sizes, the results of the within-subject univariate tests are presented using the Greenhouse Geisser estimate, which does not assume sphericity, and corrects the degrees of freedom of the $F$ statistic as a function of sphericity violations.

5. Follow-up tests were evaluated using the LSD adjustment for multiple comparisons.

6. Homogeneity of the variance-covariance matrices was evaluated at the $p < .05$ level using Levene’s estimate; this assumption was not violated unless noted.

7. Univariate outliers were identified as values that were greater than 2 SD from the group mean.

8. Homogeneity-of-regression was evaluated at the $p < .001$ level and was not violated.

9. Salivary concentrations of AA (U/mL) were subjected to square-root transformations and cortisol concentrations (µg/dL) to logarithmic transformations. The transformed values were utilized in all statistical analyses, but the actual concentrations are presented in figures for descriptive purposes.
References


Critchley, H. D., Daly, E. M., Bullmore, E. T., Williams, S. C. R., Van Amelsvoort, T.,


analysis of wakefulness and REM sleep in high functioning autistic spectrum disorders.


DeZeeuw, C. I., Hoogenraad, C. C., Koekkoek, S. K. E., Ruigrok, T. J. H., Galjart, N., &


pupil reaction to light in Alzheimer’s disease patients: A preliminary report.

*International Journal of Psychophysiology*, 37, 111 – 120.


Hadnezar, M. M., Buchsbaum, M. S., Wei, T-C., Hof, P. R., Cartwright, C., Bienstock, C.


_Acta Neurologica Scandinavica_, 88, 123 – 128.


_Developmental Brain Dysfunction_, 7, 93 – 103.


Herman, B. H., Arthur-Smith, A., Hammock, M. K., & Josephs, S.  (1988).  Ontogeny of B-


Hoshino, Y., Kumashiro, H., Kaneko, M., Numata, Y., Honda, K., Yashima, Y.,…Wantanabe,


Possible involvement of orexin in the stress reaction in rats. *Biochemical and Biophysical Research Communications*, 270, 318 – 323. doi: 10.1006/bbrc.2000.2412


Lowenstein, O., & Loewenfeld, I. E. (1961). Influence of retinal adaptation upon the pupillary


Marinovic-Curin, J., Marinovic-Terzic, I., Bujas-Petkovic, Z., Zekan, L., Skrubic, V., Dogas, Z.,


International Journal of Psychophysiology, 52, 77 – 86. doi:
10.1016/j.ijpsycho.2003.12.005


Appendix A

Social Stimulus Examples
Appendix B

Non-Social Stimulus Examples
Appendix C

*Calibration Array*
Appendix D

Developmental Disability Organizations that Assisted in Participant Recruitment

ABC’nD Enterprises LLC
Autism Alliance of Kansas City
Autism and Behavior Consulting, INC
Autism Society of America, Johnson County
Autism Society of Johnson County
Autism Speaks
Capper Foundation
Center for Child Health and Development at the University of Kansas Medical Center
Children’s Mercy Hospital
Children’s TLC
Down Syndrome Guild of Greater Kansas City
Early Childhood Autism Program (ECAP)
Individualized Behavior Solutions
Infant and Toddler Services of Johnson County
Infant and Toddler Services of Leavenworth County
Infant and Toddler Services of Topeka KS
Infant and Toddler Services of Wyandotte County
Families Together Inc. Kansas City
Families Together Inc. Topeka
Kansas City Autism Training Center
Lawrence Autism Society
Olathe School District
Successful Sounds
Sunflower Early Education Center
Tiny K Early Intervention
Appendix E

Recruitment Letter

RESEARCH AT THE UNIVERSITY OF KANSAS
Children with *Autism*, children with *Down syndrome*, and *typically developing* children are needed to participate in an autism research project at the University of Kansas.

Dear Parents:

Autism is a neurological disorder that is marked by impaired development in social interaction and communication and a restricted repertoire of activities and interests. Although we know that autism is a neurological disorder, the root of the neurological dysfunction is unknown. Therefore, research efforts have been focused at trying to identify what brain areas may primarily contribute to the disorder. In addition, because children with Down syndrome share some of the language delays with children with autism, we are interested in determining whether or not children with this diagnosis share similar neurological dysfunctions with children with autism. Hopefully, through various research efforts, we will obtain a clearer idea about the causes of autism and Down Syndrome. The research project that we will be conducting will study the function of two brain regions to determine if either of these areas may be a key to identifying and diagnosing children with autism and/or children with Down syndrome.

**What is the purpose of this research project?**
The purpose of the research project is to determine if locus coeruleus and/or hypothalamic activity is dysfunctional in children with autism and/or children with Down syndrome. The locus coeruleus and hypothalamic systems are involved in controlling pupil size and are involved in the control of attentional arousal. Therefore, this project will use activities that elicit this attentional responses and will examine salivary measures of these systems.

**Who may participate in this project?**
We are looking for children who are between the ages of 2 and 6 years of age and who have either (a) a diagnosis of *autism or PDD-NOS*, (b) diagnosed *Down syndrome* without an autism spectrum disorder diagnosis, and (c) *typically developing children*. The children must not have neurological disorders (beyond autism or Down syndrome), should be free of hearing or vision difficulties (corrected vision and hearing with glasses and/or hearing aids is okay) or other serious health problems such as heart disease. In addition, the children should not be taking any medications (vitamins are okay).

**What type of activities will my child participate in?**
Each child will be seen at our Lawrence laboratory for two separate sessions. During each session, your child will complete a visual stimuli task, in which your child will sit in a car seat and will be shown a movie clip. While they are looking at the pictures, their eye movements and pupil diameters will be recorded. In addition, salivary samples will be taken from your child at various specific time...
Appendix E (continued)

points during and after the movie clip. Finally, a standardized intelligence assessment, and a
standardized assessment of autism will be completed (they will be completed at separate sessions).
The standardized intelligence assessment will consist of a variety of activities that your child will
be asked to complete. The standardized assessment of autism will be completed while your child
participates in free play with toys.

How long will these activities take?
Participation would require 2 sessions, and each session should take approximately 2 hours to
complete. In addition, you will be asked to take salivary measures from your child on two additional
days in your home-environment, and these samples will be picked up by a research assistant when
completed.

Will we be reimbursed for our time and travel?
YES! You will be give $100 for participation in this study, $50 for each of the two sessions.

How do I sign up for participation?
If you are interested in participating in this research project or if you have any further questions,
please contact us directly at our Lawrence office at (785) 312-5345 (please ask to speak with
Christa Anderson, or leave a message if no one is available) or e-mail Christa at cjanders@ku.edu.

We hope that you will consider participation.

Sincerely,

Christa J. Anderson, M.A. and John Colombo Ph.D.
Doctoral Student
University of Kansas
Schiefelbusch Life Span Institute
KU Infant Cognition Lab
INTRODUCTION

The Scheifelbusch Institute for Life Span Studies at the University of Kansas supports the practice of protection for human subjects participating in research. The following information is provided for you to decide whether you and your child wish to participate in the present study. You may refuse to sign this form and not participate in this study. You should be aware that even if you agree to participate, you and your child are free to withdraw at any time. If you and your child do withdraw from this study, it will not affect your or your child’s relationship with this unit, the services it may provide to you or your child, or the University of Kansas.

PURPOSE OF THE STUDY

The purpose of our research is to record certain types of eye movements and ocular responses in infants who have a sibling with a diagnosed Autism Spectrum Disorder.

PROCEDURES

To do this, we will need to conduct three different assessments:

1. We will need to determine the mental ability for all of the children in this study, and so we will be administering a standardized assessment of intelligence. This should take approximately forty-five minutes.

2. We will need assess autism symptomology by administering a standard assessment of autism. This should also take approximately fifteen minutes to one half hour. In addition, while your child is completing this assessment he/she will be videotaped for blind coding purposes.

3. Finally, we will be administering a visual stimuli task. During this task your child will sit alone in a car/booster seat facing a computer screen. Eight static photographs of human faces, animal faces, toys, and landscapes will be presented to your baby for 15 to 30 seconds each. While your child is looking at the pictures, we will measure your child's pupil diameter, and measure the part of the picture at which they are looking. The eye movement measurement system uses an infrared light source that tracks your child's pupil, and a head tracking sensor that will be attached to a head band worn by your child. In addition, while your child is completing this task, he/she will be videotaped in order to monitor their behavior. This will be done at our laboratory facility, and should take approximately 1 hour to complete.

At the end of the session, we will be able give you a brief description of your child's performance, and ask that you fill out a questionnaire that pertains to your child’s health, background, and environment. You will also be asked to take 8 salivary measures from your child in their home environment on two additional days to determine how their levels of alpha-amylase and cortisol vary throughout the day and fill out a questionnaire asking about salivary collection times, food intake, physical activity, and abnormal events that may have happened that day. In addition, you will be asked to complete the Children’s Sleep Habits Questionnaire at home and return to us when completed.
Appendix F (continued)

RISKS
Please be assured that none of our procedures will present any risk to you or your child. The use of infrared light will be used to measure eye movement and pupil diameter. However, the level of the infrared light used (0.8 mW/cm²) is well below the standards for risk from infrared light sources prescribed by OSHA (10.0 mW/cm²).

BENEFITS
Upon completion of the entire project, we will send you a general report of our results. In addition, if we suspect that your child is showing symptomology indicative of autism, you will be referred to a diagnostic clinic for further assessment. You and your child’s participation will make an important contribution toward our understanding of autism.

PAYMENT TO PARTICIPANTS
Participants will be paid $50 per session for their time and travel. You do not have to consent to the study to be reimbursed; if after reading this consent you choose not to participate, you will still be given $50 for time and travel. However, parents must provide all information on the payment receipt (parent name, address, and parent social security number) to be given payment, as this is information that the University of Kansas is required to provide to the IRS. This is taxable income and you are required to report this to the IRS. Receipts will be given to the LifeSpan Institute accounting department and our lab will keep copies of these receipts in a locked file cabinet in a locked room; this will only be accessible by member of our lab.

INFORMATION TO BE COLLECTED
To perform this study, researchers will collect information about you and your child from the questionnaire that you will be asked to complete. In addition, to receive reimbursement for time and travel parents will be asked to complete a receipt which requires the parent to provide their social security number. Also, information will be collected from the study activities that are listed in the Procedures section of this consent form.

It is our policy to protect the confidentiality of all of our participants. You and your child’s name will be coded by a confidential number and will not appear in any analyses or publications involved with this study. We would also like to assure you that you and your child’s participation is voluntary and that you and your child may withdraw from the study at any time, even after you have signed this consent form. Also, you and/or your child’s decision to participate or withdraw from the study will not affect or influence any relationship that you or your child might have with our department in the future.

The information collected about you and your child will be used by: Christa Anderson, M. A, John Colombo, Ph.D., and other research members of the Infant Cognition Lab, KUCR, and officials at KU that oversee research, including committees and offices that review and monitor research studies. In addition, Christa Anderson or Dr. John Colombo may share the information gathered in this study with investigators at the University of Kansas involved in the Center for Behavioral Neuroscience in Communicative Disorders.

The researchers will not share information about you or your child with anyone not specified above unless required by law or unless you give written permission.

Permission granted on this date to use and disclose you and your child’s information remains in effect indefinitely. By signing this form you give permission for the use of you and your child’s information for the purposes of this study or any future analysis that uses the information collected during this study at any time in the future.

REFUSAL TO SIGN CONSENT AND AUTHORIZATION
You are not required to sign this Consent and Authorization form and you may refuse to do so without affecting your right to any services you are receiving or may receive from the University of Kansas or to participate in any programs or events of the University of Kansas. However, if you refuse to sign, you cannot participate in this study.
CANCELLING THIS CONSENT AND AUTHORIZATION

You may withdraw your consent to participate in this study at any time. You also have the right to cancel your permission to use and disclose information collected about you and your child, in writing, at any time, by sending your written request to: Christa Anderson or Dr. John Colombo at 1315 Wakarusa, Lawrence, KS 66049. If you cancel permission to use you and your child’s information, the researchers will stop collecting additional information about you and your child. However, the research team may use information that was gathered before they received your cancellation, as described above.

PARTICIPANT CERTIFICATION:

I have read this Consent and Authorization form. I have had the opportunity to ask, and I have received answers to, any questions I had regarding the study and the use and disclosure of information about me for the study. I understand that if I have any additional questions about my or my child’s rights as a research participant, I may call (785) 864-7429 or write the Human Subjects Committee Lawrence Campus (HSCL), University of Kansas, 2385 Irving Hill Road, Lawrence, Kansas 66045-7563, email dhann@ku.edu.

I agree to allow my child to take part in this study as a research participant. I further agree to the uses and disclosures of my and my child’s information as described above. By my signature I affirm that I am at least 18 years old and that I have received a copy of this Consent and Authorization form.

We are very grateful for your participation.

Date ___/___/___

Child’s Name________________________

Research Staff Signature  ______________________________________

Parent’s Signature ______________________________________

Parent’s Address ___________________________________________________________

[If signed by a personal representative, a description of such representative’s authority to act for the individual must also be provided, e.g. parent/guardian.]
Appendix G

Health and Background Questionnaire

University of Kansas
Autism Research Program

Health and Background Questionnaire

Child’s Date of Birth: ____/____/____

Child’s Current Health (Mark any that apply)

____ Has a cold
____ Running a temperature
____ Has an ear infection
____ Is currently taking medication (prescription or over the counter).
   Please specify: ______________________________________
____ Has taken medications (prescription or over the counter) within the last 48 hours.
____ Has had shots within one week of appointment
   If yes, what shots:
   Date of shots: ________________________________
____ Has been rehospitalized since birth
   If so, for what condition? ________________________________
   For how long?
____ Has chronic condition Please specify: ________________________________
____ Other (please indicate if there are any conditions not listed here that your child has)
   Please specify: ______________________________________

Child has had ____ ear infections since birth.

Time your child last had anything to eat (including gum or candy): _____________________________
Time your child last had anything to drink: ________________________________________________

Time your child last consumed caffeine (this includes chocolate, soda, tea, or other caffeinated products):
___________________________________________________________________________________

Time your child last engaged in exhaustive physical activity (running, swimming, biking, etc): _______
___________________________________________________________________________________

Today’s food intake:
   Please list all foods that your child has eaten today:
___________________________________________________________________________________
___________________________________________________________________________________
___________________________________________________________________________________
___________________________________________________________________________________

Traumatic events:
   Please list any events that happened today that may have been stressful or unusual to the child
   (i.e., loss of a pet, car accident, loss of a favorite toy, birthday party etc.)
___________________________________________________________________________________
___________________________________________________________________________________
___________________________________________________________________________________
Appendix G (continued)

Autism/Down’s syndrome Diagnosis:

Does your child have the diagnosis of autism?  
_______

If yes, when did your child receive this diagnosis?  
_______

At what age did you first notice autism-like symptoms in your child?  
_______

Is your child currently receiving services?  
_______

If yes, what type of services is your child receiving  
(e.g., behavioral intervention, speech therapy, etc.)?  
_______

—Please list all that apply.

____________________________________________________________________
____________________________________________________________________
____________________________________________________________________

How many hours per week is your child receiving each service?  

____________________________________________________________________
____________________________________________________________________
____________________________________________________________________

Does your child have a Down’s syndrome diagnosis?  
_______

Is your child currently receiving services?  
_______

If yes, what type of services is your child receiving  
(e.g., behavioral intervention, speech therapy, etc.)?  
_______

—Please list all that apply.

____________________________________________________________________
____________________________________________________________________
____________________________________________________________________

How many hours per week is your child receiving each service?  

____________________________________________________________________
____________________________________________________________________
____________________________________________________________________

Caregiving Arrangements (these caregiving arrangements should not include treatment services for autism or a developmental disability).

Child is in daycare for _____ hours per week. (If not in daycare, enter zero)

If in daycare, what type:  
_____ Daycare center  
_____ Home-based care  
______ Your home (i.e., you run a daycare for other children  
______ A relative’s home (e.g., grandparent, aunt, etc.)  
______ Someone else’s home  
______ Private caretaker/nanny/au pair in your home

Home Environment

How many siblings living at home full-time?  _____ (include half-siblings)  
Ages of these siblings  

How many siblings visit or live at home part-time?  _____  
Ages of these siblings  

Approximate frequency and length of visit/stay:  

_____ Individuals other than the baby’s mother, father, siblings living at home full-time  
______ grandmother  
______ grandfather  
______ aunt  
______ uncle  
______ friend  
______ other (_______________________)
Appendix G (continued)

<table>
<thead>
<tr>
<th>Age</th>
<th>High School</th>
<th>Jr. Coll.</th>
<th>College or University</th>
<th>Grad Degree (MA, PhD, MD, JD, etc.)</th>
<th>Occupation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mother</td>
<td>1 2 3 4</td>
<td>1 2</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Father</td>
<td>1 2 3 4</td>
<td>1 2</td>
<td>1 2 3 4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Appendix H

Current Health Questionnaire

University of Kansas
Autism Research Program

Current Health Questionnaire

Child’s Current Health (Mark any that apply)
_____ Has a cold
_____ Running a temperature
_____ Has an ear infection
_____ Is currently taking medication (prescription or over the counter).
    Please specify: ______________________________________
_____ Has taken medications (prescription or over the counter) within the last 48 hours.
_____ Has had shots within one week of appointment
    If yes, what shots:____________________________________
    Date of shots:_______________________________________
_____ Has chronic condition  Please specify: _______________________________
_____ Other (please indicate if there are any conditions not listed here that your child has)
    Please specify: ___________________________________________________

Time your child last had anything to eat (including gum or candy): _____________________________
Time your child last had anything to drink: ________________________________________________
Time your child last consumed caffeine (this includes chocolate, soda, tea, or other caffeinated products):
___________________________________________________________________________________
Time your child last engaged in exhaustive physical activity (running, swimming, biking, etc): __________________________________________________________________________________________

Today’s food intake:
    Please list all foods that your child has eaten today:
____________________________________________________________________________________
____________________________________________________________________________________
____________________________________________________________________________________
____________________________________________________________________________________

Traumatic events:
    Please list any events that happened today that may have been stressful to the child
    (i.e., loss of a pet, car accident, loss of a favorite toy, birthday party etc.)
___________________________________________________________________________________
___________________________________________________________________________________
___________________________________________________________________________________
___________________________________________________________________________________
Appendix I

Report Letter

Dear Parent:

We thank you for your participation and support of the University of Kansas Autism Research Project. As part of the tests administered for the Autism Research Project, we gave (child’s name) two tests. One was the Mullen, which measures various mental abilities. The other was the Autism Diagnostic Observation Schedule Module 1 or 2, which measures the degree to which autism-like symptoms are exhibited. The purpose of this letter is to give you some feedback on your child’s performance on these measures. Similar letters are provided to all of the families in the project.

Before providing this information, we think that it is important for us to emphasize that the outcome of these tests should be interpreted very cautiously. There are three reasons for this:

1. These two tests were given by our research assistants for the purposes of our research project. Our staff are not trained clinicians, and so the test results cannot be used to diagnose developmental delays or autism, or to identify children with high ability.

2. Measures of children’s ability during the toddler or preschool ages is far from an "exact science." Scores at these ages are greatly influenced by many factors that can artificially lower a child's score (the child's mood, sleepiness, and/or hunger at the time of testing) or raise a child's score.

3. These tests measure only a limited number of abilities, and do not measure many other skills or strengths that your child may have in other areas.

Mullen

For the Mullen, we obtain "percentile ranks" for the Early Learning Composite (an overall score), and for four subtests (tests of specific abilities, Visual Reception, Fine Motor, Receptive Language, and Expressive Language). The percentiles range from 99 to 0, with higher numbers reflecting more optimal scores. In addition, a descriptive category is presented next to each score. This description explains where your child’s scores lie, relative to the typically developing children that were tested in the making of the test.
Appendix I (continued)

Your child’s percentile scores on the Mullen are as follows:

<table>
<thead>
<tr>
<th>Subscale</th>
<th>Percentile</th>
<th>Descriptive Category</th>
<th>Age Equivalent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual Reception</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fine Motor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptive Language</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expressive Language</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early Learning Composite (overall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>score)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Autism Diagnostic Observation Schedule (ADOS)**

The ADOS yields two category scores (Communication and Qualitative Impairments in Reciprocal Social Interaction) which are added together to generate one total score. The scores for this test indicate the degree to which autism-like symptoms were present. The cut-off score for falling within the autism range is 12; this means that any scores below 12 do not qualify as being in the range of autism. Again, these scores are not meant to diagnose or not diagnose your child. This test was completed for research purposes only, and only a qualified clinician who would complete several assessments on your child is able to give a diagnosis.

Your child’s score on the ADOS was:  

The phrase 'no score' may appear in one or more of the boxes above. If that occurs, it means that we were unable to obtain what we would consider to be a valid score for your child on that test or subtest.

If you have any questions or concerns, please feel free to call the laboratory at (785) 312-5345. Again we would like to thank you for your participation in our project.

Sincerely,

Christa J. Anderson, M.A. and John Colombo Ph.D.
Doctoral Student
University of Kansas
Schiefelbusch Life Span Institute
KU Infant Cognition Lab
Appendix J

Non-Social Stimulus Example with Defined Look Zones (White Lines)
Appendix K

Social Stimulus Example with Defined Look Zones (White Lines)

Internal features: Represented in the shaded area
External features: Area within white lines, excluding shaded area

Hands: Represented in the shaded area
Body: Area within white lines, excluding shaded area